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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

A multidisciplinary project is underway to study the influence of low-level exposure to chemicals to which military personnel had contact during the Persian Gulf War. The research group includes investigators with expertise in neuroscience, molecular biology, enzymology, cardiovascular physiology and neuropharmacology. The focus is on testing the effect of low doses of pyridostigmine bromide (PB), DEET, and Sarin with investigations of 1) effect of combined chemical and stress exposure on behavioral, cardiovascular, endocrine and cholinergic function 2) effect of stress and chemical exposure on auditory brain responses using electrophysiology, energy metabolism using nuclear magnetic resonance (NMR) techniques and mitochondrial function 3) activity of aldehyde and alcohol dehydrogenases and esterases in human samples with the goal of establishing whether there are alterations in populations characterized as chemically sensitive and 4) gene expression using a DNA microarray system to test the effect of chemical exposure on a neuronal cell line. The overall objective is to study the effect of chemical exposure from the single cell/gene level to the whole animal and ending with the human condition.

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INTRODUCTION

A multidisciplinary project is underway to study the influence of low-level exposure to chemicals to which military personnel had contact during the Persian Gulf War. The research group includes investigators with expertise in neuroscience, molecular biology, enzymology, cardiovascular physiology and neuropharmacology. The focus is on testing the effect of low doses of pyridostigmine bromide (PB), DEET, and Sarin with investigations of 1) effect of combined chemical and stress exposure on behavioral, cardiovascular, endocrine and cholinergic function 2) effect of stress and chemical exposure on auditory brain responses using electrophysiology, energy metabolism using nuclear magnetic resonance (NMR) techniques and mitochondrial function 3) activity of aldehyde and alcohol dehydrogenases and esterases in human samples with the goal of establishing whether there are alterations in populations characterized as chemically sensitive and 4) gene expression using a DNA microarray system to test the effect of chemical exposure on a neuronal cell line. The overall objective is to study the effect of chemical exposure from the single cell/gene level to the whole animal and ending with the human condition.

BODY

The details of the research accomplishments will be organized according to the research programs as described in the introduction.

Project 1. The effect of combined chemical and stress exposure on behavioral, cardiovascular, endocrine and cholinergic function.

Project 2. The effect of stress and chemical exposure on auditory brain responses, energy metabolism and tissue chemical constituents in an animal model.

Project 3. The study of enzymes involved in chemical metabolism, activity of dehydrogenases and esterase in human tissues with the goal of establishing whether there are alterations in populations characterized as chemically sensitive.

Project 4. The study of gene expression using a DNA microarray system to test the effect of chemical on a neuronal cell line.

During the second year of the project, the focus has been on conducting studies related to the effects of pyridostigmine, DEET and stress on organistic and cellular systems. Another major goal of the contract is the investigation of the effects of chronic low-dose Sarin exposure using multiple endpoints such as tissue metabolism, gene expression, animal behavior, and central nervous system function. For this reason, a specialized laboratory was constructed for the use of dilute CWA. Although the laboratory was completed in year 1, there have been delays in its implementation. This has resulted from problems in laboratory approval, availability of Sarin, and certification for use. Some of the delay can be attributed to the consequences of the terrorist attacks in September, 2002, the military deployments and the heightened security. For example, before this time it was possible to receive dilute Sarin solutions via Federal Express; whereas,

afterwards, a military courier was required for delivery. This resulted in a delay in receiving the dilute CWA, delivery in May, 2002 after ordering it in February, 2002. Another issue was the acquisition of chemical bubble testers for monitoring the effectiveness of the hood system. There was a lag time of almost 4 months before the testing devices were received at WSU. The last step is the analysis of the bubble testers and the final certification that the facility is ready for use. We are confident that this will occur within the next few weeks. The Sarin is in hand under storage at - 80° C. All of the safety plans are in place as is the safety equipment. This work has been performed under the able direction of Drs. James Lucot and Sara Paton in the Department of Pharmacology and Toxicology. We have also been assisted by Benjamin Casole, Chief Safety and Chemical Officer, US Army Medical Research Institute of Chemical Defense and Dr. Jerry Hagan, Chief, Office of Environmental Health and Safety, Wright State University.

Project 1: Studies of combined chemical and stress exposure on behavioral, endocrine, cholinergic, and cardiovascular function.

The investigators are Morris, Lucot, Cool, Grubbs and McDougal. The research personnel are Bernatova, Dubovicky, Mach, Paton, Price and Ropp. There is a general collaborative arrangement between the investigators with the lead person for each of the groups as follows; Behavior (Lucot), Cardiovascular/Endocrine (Morris), Cholinergic Systems (Grubbs), Proteomics and Confocal Microscopy (Cool). McDougal, Professor of Pharmacology and Toxicology at WSU and Director of Toxicology Research, has been recruited to the researchteam. He is an expert in chemical toxicology and genomics/proteomics and will interface with all the groups. McDougal replaces Guneseckar who was terminated because of geographical relocation.

Experimental Model: The research aims in project 1 focus on stress/chemical interactions, as such, the investigations use the same animal model. This model combines chronic shaker stress with chemical exposure. The group collaborates on the experimental testing with the specific protocols designed for the needs of the individual investigators. The stress model was developed and tested in year 1 with publications forthcoming on its characterization (Bernatova et al. and Dubovicky et al.). The basic design uses C57Bl adult male mice exposed to intermittent shaker stress for a 7 day period. The animals are housed in a specialized caging system which is attached to the shaking platform. The timing of the shaking (150 cycles/min with a horizontal excursion of 2.86cm) is regulated by a computer. The animals are exposed to 45, 2 min shaking sessions/24 hr with an average inter-stress interval of 30 min. The test compounds (saline or pyridostigmine bromide – PB) were administered by osmotic minipump implanted sc under the back of the mouse (2 ul/min).

BEHAVIORAL SYSTEMS

Investigators Jim Lucot, Ph.D., Michal Dubovicky, Ph.D. and Mojmir Mach, M.S.

The focus of work in the second year was on the study of the effects of chronic stress exposure, PB or a combination of both factors on behavior of mice. The experiments used a variety of behavioral tests, including, acoustic startle, pre-pulse inhibition, open field, and elevated plus maze. We also evaluated the influence of the circadian cycle (light-inactive/dark-active) on the behavioral responses. The stress model is chronic intermittent shaker stress (see Introduction to Project 1) with the drugs administered via osmotic minipump (sc).

KEY ACCOMPLISHMENTS:

- Work was completed on characterization of the chronic stress model. The results indicate that intermittent shaker stress provides a good model for chronic stress exposure in mice.
- Results show that there are time dependent changes in the behavioral responses to chronic stress. Differences were associated with the light period in which the animals are less active. This data is complementary to the cardiovascular results which show a similar circadian variation. There were no overall effects of stress on ASR or locomotor activity
- Acute shaker stress produced no behavioral changes.
- Completed studies on Stress/PB interactions in mice.
- Studies of PB exposure show that PB at doses lower than 10 mg/kg failed to evoke any behavioral changes in mice.
- PB administered at 10 mg/kg/day resulted in exaggerated ASR and inhibition of locomotor activity in the open field without any prolonged or delayed effects. This suggests that PB has central neural effects when given chronically to mice.
- Interactions between chronic stress and PB were observed. Stress inhibited the exaggerated ASR produced by PB administration.
- Presentations were made at national meetings (Society of Toxicology and Biosciences Review), documenting the results on stress/PB interactions in mice.
- Two manuscripts were submitted and others are in preparation.

1. Effect of acute shaker stress on the behavior of mice in the open field and elevated plus maze

The stress paradigm uses 2 min periods of shaking, administered over 7 days. An experiment was conducted to evaluate the effect of a 2 min acute stress on behavior of mice. Mice were assigned into the experimental groups based on their whole body movement (controls and acute stress, n = 6/group). Three days later they were acutely stressed and tested in the open field in a 15 min session. The times were: immediately after the stress, than 7 and 14 days thereafter. There were no significant differences between controls and acutely stressed mice for any of the individual variables of open field behavior. A second experiment tested the effect of a longer acute stress (15 min). There were no significant effects of a 15 min acute stress on open field behavior of mice. In addition, acute stress had no significant influence on behavior in the elevated plus maze. The acute stress data was presented as an abstract at the SOT meeting and the WSU Stress Symposium. It is also included in a paper submitted to Physiology and Behavior (Dubovicky et al)

2. Effect of PB and physostigmine (PHY) on open field behavior of mice

In this series of experiments, we tested effect of 4 doses of PB (0, 1, 3, 6 and 10 mg/kg/day) on behavior of mice in the open field. PHY (2.88 mg/kg/day) was used as a positive control as it is known to cross the blood brain barrier and inhibit locomotor activity in experimental animals. Mice were tested in the open field in 15 min session before treatment and on day 6 of the drug administration. There was a trend for PB to inhibit locomotor activity in the open field. However, due to small number of animals (n = 5/group) and big inter-individual differences, the change was not significant. PHY administration caused a significant decrease in locomotor activity compared to sham-operated mice ($p < 0.05$, MANOVA; Table 1-1).

Table 1-1. *Effect of PB and PHY on locomotor activity of mice after 6 days of sub-acute treatment. *p < 0.05, significant difference compared to sham-operated controls*

Groups	Locomotor activity (activity counts)
Sham	1919.33 ± 121.77
PB 1 mg/kg	1751.17 ± 165.42
PB 3 mg/kg	1622.83 ± 126.25
PB 6 mg/kg	1717.67 ± 69.88
PB 10 mg/kg	1624.67 ± 60.01
PHY 2.88 mg/kg	1541.67 ± 113.75 *

3. Effect of PB on behavior of mice in the elevated plus maze

Mice were treated with PB using osmotic minipumps (0 or 10 mg/kg/day). Separate groups of mice were tested during the dark phase on days 3 and 7 of PB administration (n = 20/group). Mice were tested once in the elevated plus maze in a 5 min session in the dark (active) phase of the circadian rhythm (1 hour after lights off, testing room illuminated by red lights). Testing in the elevated plus maze revealed no significant effect of PB on the behavior of mice on both days of treatment compared to sham operated controls (data not shown).

4. Effect of PB and chronic stress on the acoustic startle response (ASR) and prepulse inhibition (PPI).

Mice were treated with PB (10 mg/kg/day) or saline using osmotic minipumps and exposed to no stress or chronic stress (n = 20/group). The PB and stress were administered for 7 days. Mice were tested in the SM100 Startle Monitor System Version 4.0 (Hamilton Kinder, 2001, Poway, CA). The system was set up for 6 types of stimulus trials: no stimulus (background, 60 dB), pre-pulse (70 dB), pulse (100 dB and 120 dB), pre-pulse plus pulse (70 dB+100dB and 70 dB+120 dB). Each trial type was presented 10 times in 10 blocks. Stimuli were presented in random order to avoid order effects and habituation. The inter-trial interval varied from 9 to 16 sec. Mice were pair matched according to baseline values into four groups: sham-operated (SH), sham-operated plus stress (SH+S), pyridostigmine (PB) and pyridostigmine plus stress (PB+S). Mice were tested during chronic stress on days 2 and 7 (S2, S7) and days 7,

14, 21 and 28 days (D7, D14, D21, D28) after discontinuation of treatment. We used the extended time course in order to determine whether there were any long term or delayed effects as observed with other models..

The acoustic startle responses for both 100 dB and 120 dB stimuli were significantly higher in the PB group on days 2 and 7 of PB and stress exposure compared to sham-operated controls ($p < 0.01$, MANOVA). Similarly, the mean values of ASR in PB+S mice were increased compared to SH+S controls. However, this increase failed to be statistically significant. There was no evidence for delayed effects in any of the experimental groups (Fig. 1-1 and Fig. 1-2). Pre-pulse inhibition processes were not affected by PB treatment, stress or the combination of these factors (data not shown). The results show that PB increased the ASR in mice, suggesting some central neural interaction between the drug or its effector mechanisms. The combination of stress and PB was not different from Sham, indicating that stress may modulate the response.

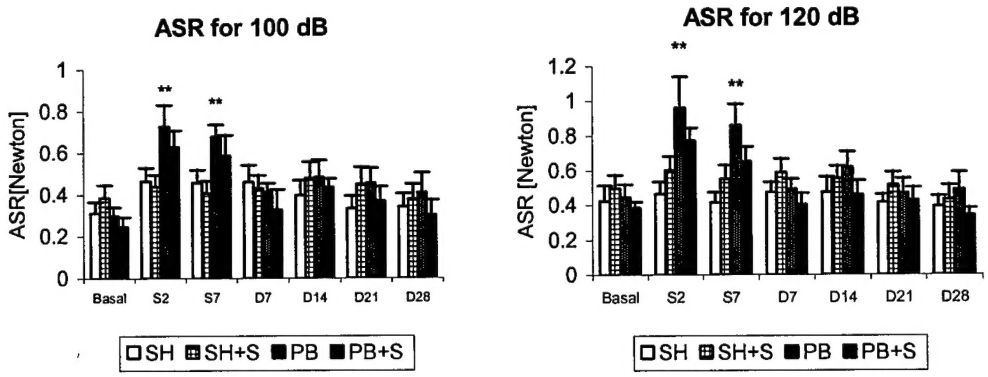


Figure 1-1 and Figure 1-2 *Effect of PB treatment on acoustic startle response in mice.* $**p < 0.01$*, MANOVA, significant differences compared to sham-operated controls.*

5. Influence of the circadian cycle on stress-induced behavioral responses.

Mice were assigned into groups according to their baseline level of locomotor activity. Three days later, they began either 7 days of chronic stress exposure (shaker stress, n = 20) or a single 15 min period of acute shaking (acute stress, n = 18). Control mice (20 mice) were housed in their home cages, except for the time allocated to behavioral testing. Behavioral tests were conducted immediately after the end of the 7 day stress (Day 1) and on days 7, 14 and 21 after termination of the stress. The long time course was used in order to test for delayed effects. Mice were exposed to the open field in 15-min sessions once daily in the same time schedule between 0900-1300 (light phase) and 1 hour after lights off (1800 - 2100, dark phase, red light illumination).

There were no differences in baseline locomotor activity between mice tested under different light conditions. However, there were differences in the stress-induced changes between the light (inactive) and dark (active) periods. In mice tested in the light phase, chronic stress, but not acute stress, resulted in a decrease in locomotion. ($p < 0.01$, MANOVA, Fig. 1-3). Neither chronic nor acute stress evoked any other changes in the behavior of mice (Fig. 1-5).

However, both chronically and acutely stressed mice tested under dark conditions immediately after stress had lower emotional reactivity (evaluated using fecal boli) compared to controls ($p < 0.05$, MANOVA, Fig. 1-6). The results indicate higher sensitivity of chronically stressed mice exposed to a new environment in the light phase of the circadian cycle.

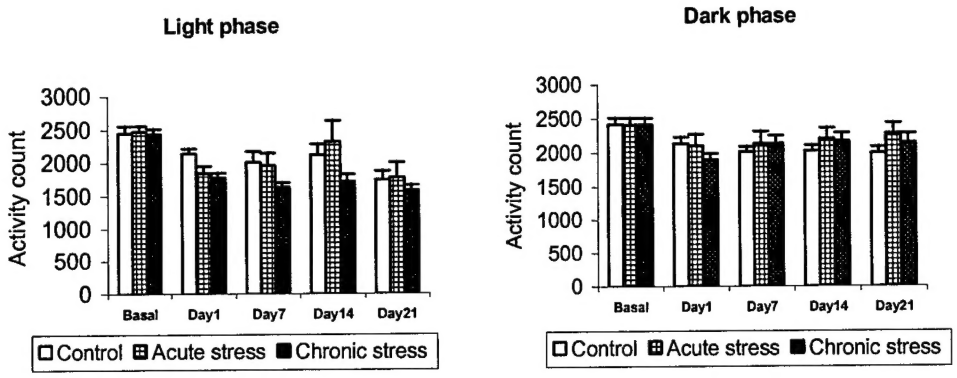


Figure 1-3 and Figure 1-4 *Effect of sub-acute shaker stress on locomotor activity of mice in the dark and light phase. Main effect of sub-acute stress in the light phase, $p < 0.01$, MANOVA.*

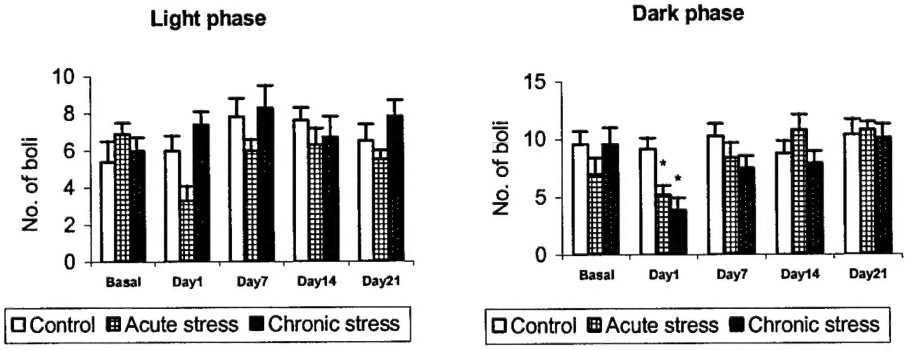


Figure 1-5 and Figure 1-6 *Effect of sub-acute shaker stress on emotional reactivity in mice in the dark and light phase. Main effect of sub-acute stress in the light phase, * $p < 0.05$, MANOVA.*

6. Effect of PB in interaction with chronic stress on open field behavior

Mice were assigned into the experimental groups according to baseline values of their locomotor activity (sham, PB, sham + stress, PB + stress, n = 10). They were treated with PB (10 mg/kg/day) or saline and exposed to no stress or 7 days of intermittent shaker stress. They were tested in the open field in 15-min session daily on days 1, 3 and 6 of treatment. Mice treated with PB had lower locomotor activity during the experiment. There was a significant main effect of PB treatment on locomotor activity of mice ($p < 0.01$, MANOVA). However, post hoc test did not reveal any significant changes on individual days of testing (Figure 1-7).

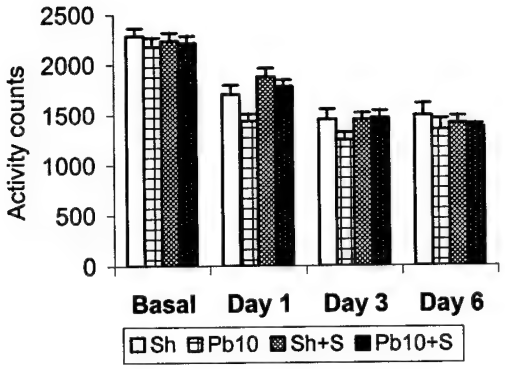


Figure 1-7. *Effect of PB treatment and shaker stress on locomotor activity of mice. Significant main effect of treatment, $p < 0.01$ MANOVA.*

Presentations and Publications:

Presentations were made at the Society of Toxicology Meeting, the Earl H. Morris Symposium "Stress: Adaptation vs Disease" held on the WSU campus and the Biosciences Review. Dr. Lucot will attend the Society of Neurosciences Meeting and give a presentation in November, 2002 and Dr. Dubovicky will attend the International Stress Symposium. Two papers have been submitted which provide data on the characterization of the model and the circadian variation in stress responsiveness (Dubovicky et al., Physiology and Behavior and Bernatova et al., Hypertension). Two manuscripts are in preparation.

Abstracts (see Appendix):

M. Bernatova, M. Dubovicky, M. Key, J.B. Lucot, M. Morris: Chronic stress alters cardiovascular and endocrine responses in mice. The FASEB Journal 16: A506, 2002.

Bernatova, M. Dubovicky, S.J. Paton, J.B. Lucot, M. Morris: Pyridostigmine treatment alters stress responsiveness in mice. Bioscience Review. US Army Medical Defense. Abstract Program Book. P123, 2002.

M. Dubovicky, I. Bernatova, M. Morris, J. B. Lucot: Development of a model for chronic stress exposure in mice. The Toxicologist 66, 252, 2002.

N. Jackson, J.B. Lucot: Stress alters the motor and neurochemical responses to MPTP. Earl H. Morris Symposium, "Stress: Adaptation vs Disease," 2002.

J.B. Lucot, M. Dubovicky, J.R. Wells: Effect of pyridostigmine and chronic shaker stress on acoustic startle response, pre-pulse inhibition and open field behavior of mice. Earl H. Morris Symposium, "Stress: Adaptation vs Disease," 2002.

J.R. Wells, M. Dubovicky, J.B. Lucot: Effects of shaker stress on the open field behavior of mice under diurnal and nocturnal conditions. International Stress Symposium 2002.

Publications:

Bernatova, M.P. Key, J.B. Lucot, M. Morris: Circadian Differences in Stress-Induced Pressor Reactivity in Mice, In Revision, Hypertension, 2002.

M. Dubovicky, J.R. Wells, M. Morris, J. B. Lucot: Chronic shaker stress alters day/night behavioral patterns in mice. Submitted Physiology and Behavior, 2002.

I. Bernatova, M. Dubovicky, J. B. Lucot, M. Morris: Effect of pyridostigmine treatment on behavioral and cardiovascular parameters in mice. in preparation for Toxicological Sciences.

M. Dubovicky, J.R. Wells, S. Paton, B. Mauck, M. Morris, J.B. Lucot: Effects of chronic shaker stress and pyridostigmine treatment on acoustic startle response, pre-pulse inhibition and open field behavior in mice. In preparation for Toxicological Sciences.

CHOLINERGIC SYSTEMS

Investigators: Robert Grubbs, Ph.D., Brena Mauck, M.S. and William Price, B.S.

KEY ACCOMPLISHMENTS:

- Tested and validated the chronic stress/PB exposure model in mice from the perspective of effects on cholinesterase activity in blood and different brain regions.
- Initiated studies to assess the effect of PB and stress on apoptosis in brain.
- Initiated studies to assess the effect of stress and PB on muscarinic receptor density in brain
- Gave presentations at the Society of Toxicology Annual Meeting, Biosciences Review and Toxicology Risk Assessment Meeting (sponsored by the EPA).

Validation of the model:

The experimental model uses Alzet™ minipump implanted subcutaneously for the constant infusion of PB in mice. The drug is given in a slow infusion over a 7-day period. The first step was to evaluate the effect of PB administration on blood AChE levels. Results show that PB infusion produces a reproducible inhibition of blood cholinesterase activity as expected. To achieve our targeted 30-40% inhibition of blood cholinesterase activity, we determined that a dose of 10 mg/kg PB was needed. While this dose is considerably higher than what we had expected to use, it apparently reflects the absorption kinetics of this route of slow sc administration in mice. The same dose was lethal when given acutely iv to mice. Chronic intermittent shaker stress alone produced no changes in blood or brain AChE activity.

The effect of PB on brain cholinesterase activity appears to depend on the specific brain area assayed and on whether the animal has been stressed. For example, while the frontal cortex appears to be insensitive to PB alone, exposure to stress and PB induced a small but statistically significant inhibition of CHE activity. In contrast, analysis of brain stem from animals exposed to 10 mg/kg PB revealed a significant reduction ($P<0.03$) in BChE activity regardless of whether the animal had been stressed, while AChE activity levels remained unchanged. However, we also noted that BChE activity increased significantly ($P< 0.02$) in sham controls relative to non-implanted controls. These observations are of particular interest in view of the recent work conducted with AChE knockout mice indicating a previously unrecognized and potentially important role for BChE in the brain (Li et al., 2000; Mesulam et al., 2002).

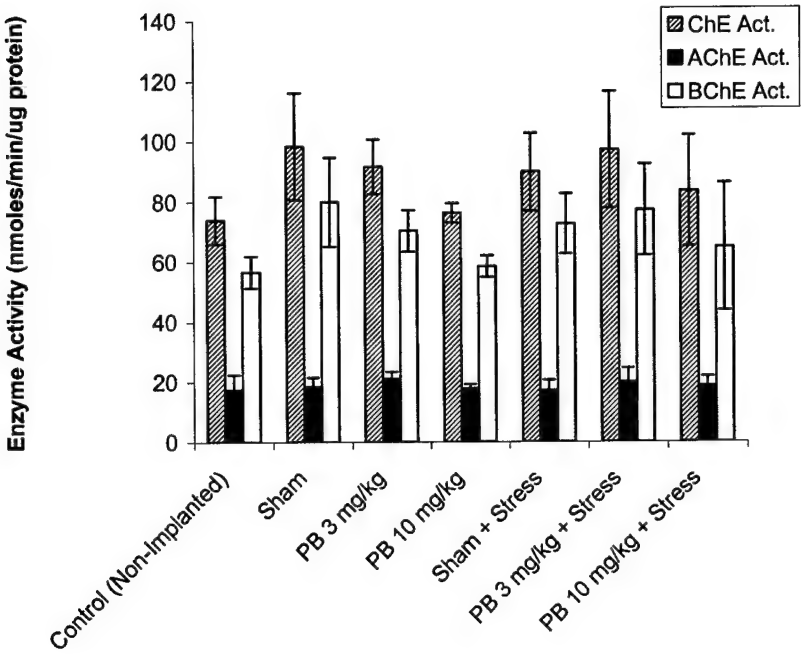


Figure 1-8. Effects of stress and PB on cholinesterase activity in mouse brain stem. Animals were exposed to PB and/or stress for 7 days. Sham denotes animal underwent surgery with no pump implanted.

Effect of PB and stress on apoptosis in brain:

An initial survey of brain tissues on day 7 and day 37 after one week of shaker stress and exposure to pyridostigmine showed that apoptotic cells are present at both time points but in different tissues. On day 7 apoptosis is clearly present in hippocampus but not cerebellum (Figure 1-9). On day 37 apoptosis is present in cerebellum but not in hippocampus. The analysis of each tissue is continuing.

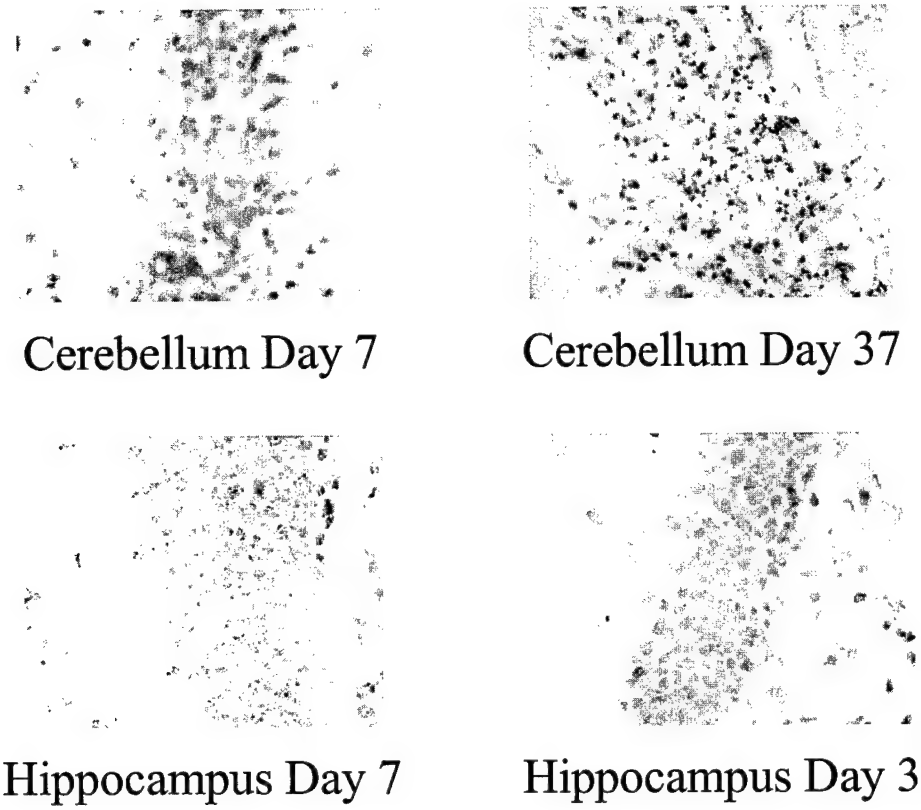


Figure 1-9. Stress and PB exposure for one week induce apoptosis at different times in the cerebellum and hippocampus. Apoptotic nuclei are stained brown (black in diagram) by the chromogenic substrate (diaminobenzamine) and counterstained with a proprietary Blue Counterstain that indicates the total number of cells present.

Effect of stress and PB on muscarinic receptor density in brain:

We are in the midst of analyzing two experiments worth of autoradiographic data to determine whether there are changes in muscarinic receptor density in several brain areas following 7 days exposure to PB and/or stress. The pseudocolor images shown in Figure 1-10 illustrates the specificity of binding (inhibition by atropine) and the inherent regional differences in muscarinic receptor density. The highest densities are seen in the caudate followed by the cerebral cortex.

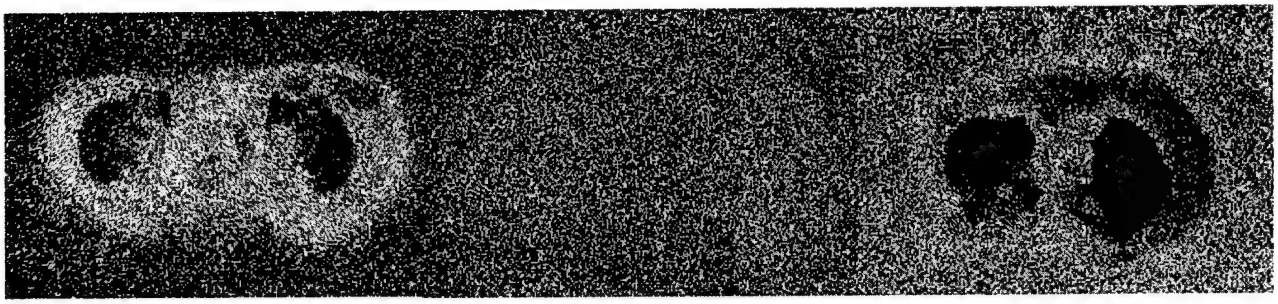


Figure 1–10. Autoradiographic images of mouse brain sections labeled with [^{3}H] N-methyl-scopolamine with (center) and without (side panels) 1 uM atropine.

Presentations and Publications:

Abstracts (See Appendix):

Grubbs, RD, WA Price, BS Mauck, I Bernatova, SJ Paton, DR Cool, JB Lucot, M Morris. Cholinesterase Activity in Mice Chronically Exposed to Pyridostigmine Bromide. The Toxicologist 66: 252, 2002. Presentation at the 2002 Society of Toxicology meeting.

Grubbs, RD, WA Price, BS Mauck, I Bernatova, SJ Paton, DR Cool, JB Lucot, M Morris. Cholinesterase Activity in Mice Chronically Exposed to Pyridostigmine Bromide. Abstract/poster presentation at the Theories and Practices in Toxicology and Risk Assessment Conference in Cincinnati, OH, 2002.

Grubbs, RD, WA Price, BS Mauck, SA Ropp, I Bernatova, SJ Paton, M Morris, JB Lucot, DR Cool. Regional Differences in Brain Cholinesterase Activity and Protein Expression in Mice Following Subacute Stress and Exposure to Pyridostigmine Bromide. Bioscience Review. US Army Medical Defense. Abstract Program Book. P118, 2002. Presentation at Bioscience 2002.

Publications:

Bernatova, I., M. Dubovicky, J. B. Lucot, R. Grubbs, W. Price, M. Morris: Effect of pyridostigmine treatment on behavioral and cardiovascular parameters in mice. in preparation for Pharmacology, Biochemistry and Behavior.

Proteomics
Investigators: David Cool, Ph.D. and Samuel Ropp, M.S.

The objective is to study the effect of PB and chronic stress on hypothalamus peptide/cholinergic systems using proteomic tools such as SELDI-TOF mass spectroscopy, Western blotting, acetylcholinesterase enzyme assays and real-time PCR. The end result will be a complete characterization (from mRNA to protein) of the changes in hypothalamic proteins produced by stress and chemical stimulation.

KEY ACCOMPLISHMENTS:

- Completed studies (6 experiments) on the effect of PB and stress on hypothalamic AChE activity
- Developed the methods for the Western blot analysis of AChE protein in hypothalamus or other tissues
- Used proteomic mass spectrometric methods for the analysis of hypothalamus, anterior and posterior pituitary tissue from PB/stress experiments.
- Developed the methods for the real-time PCR analysis of AChE, GAPDH and other hypothalamic peptides
- Gave presentations at the Society of Toxicology Meeting, including a poster discussion in the session on Proteomics and Toxicology
- Submitted a paper for publication to the Journal of Chromatography
- Directed the research program of Sam Ropp, a Ph.D. student working on the DoD project

Results for Specific Experiments:

1) P01A- Dose-response with 0 (sham), 0.5, 1, 2 and 3 mg/kg/day pyridostigmine Br for 7 days, after which they were sacrificed and tissue collected for analysis.
2) P01B- Repeat of P01A- except the doses were changed to, 0 (sham), 1, 3, 6 and 10 mg/kg/day pyridostigmine Br.
3) PS01A- Six mice each were infused with pyridostigmine Br, 0 (sham), 3 and 10 mg/kg/day for 7 days, in the presence or absence of random shaker stress.
4) PS01B- Repeat of PS01A- 10 mice each were infused with pyridostigmine Br, 0 (sham) and 10 mg/kg/day in the presence or absence of random shaker stress.
5) P02A- 10 mice each were treated with 0 (sham) or 10 mg/kg/day pyridostigmine Br for 1, 3 or 7 days.
6) PS02B- 10 mice were infused with 3 and 10 mg/kg/day pyridostigmine Br in the presence or absence of stress to repeat PS01A and PS01B.

Results:

a) Acetylcholinesterase Activity- Hypothalamus-

Hypothalamic lysates from the control, PB-treated and stressed mice were analyzed for acetylcholinesterase (AChE) activity. Kinetic analysis revealed that the AChE activity in the

hypothalamus was affected by PB. From the experiments, P01A and P01B, we saw a dose-dependent decrease in AChE activity in response to increasing concentrations of PB. However, when PB was increased to 6 and 10 mg/kg/day, AChE activity was found to increase in a dose-dependent manner (Figure 1-11). With the addition of stress to the PB exposure (PS01A), we saw that stress reversed the increase in AChE activity that was induced by 10 mg/kg/day (Figure 1-12).

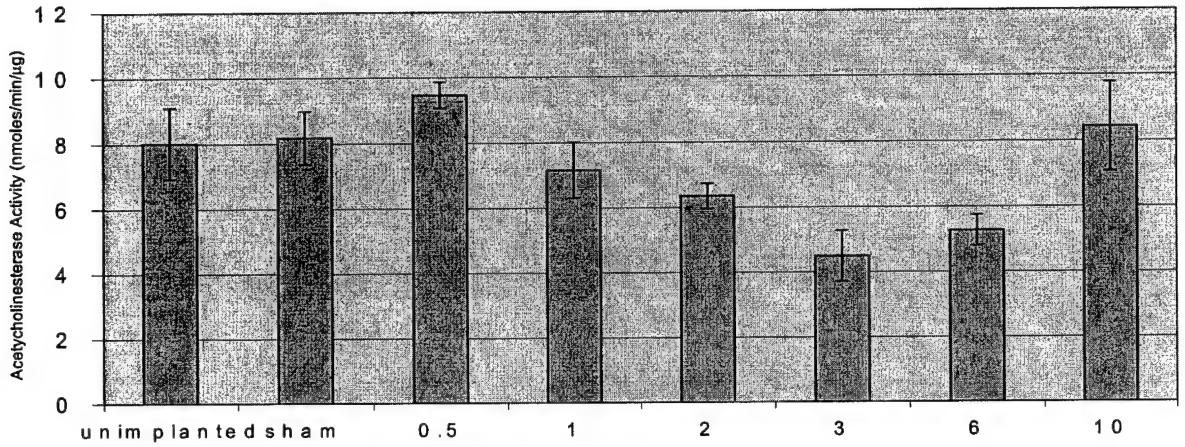


Figure 1-11. Summary of Experiments P01A & B. Hypothalamic AChE activity at 4 doses of PB, 1, 3, 6 and 10 mg/kg/day delivered by Alzet mini-pumps. (mg/kg/day- mkd). Only 3 and 6 mkd were significantly different than sham or non-implanted controls. There was also a significant difference in 3, 6 and 10 mkd, but not sham and 10 mkd.

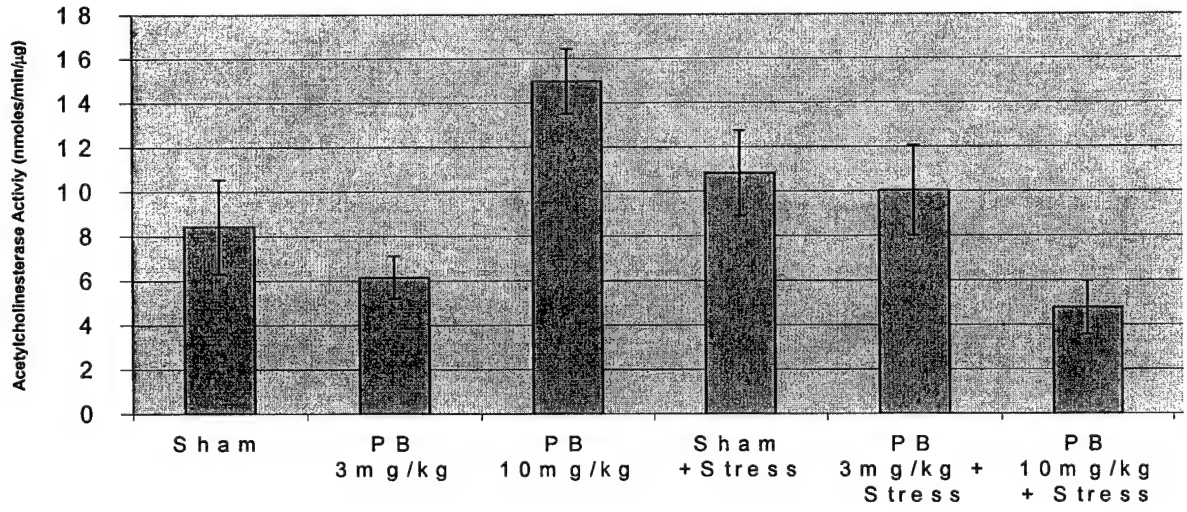


Figure 1-12. Experiments PS01A, PS01B and PS02B. Combinatorial effect of stress with pyridostigmine bromide on hypothalamic AChE activity. 10 mg/kg/day is significantly different from 3 mg/kg/day as in the previous experiment. They also indicate a difference in 3 mg/kg/day and 3mg/kg/day + stress. There also was a significant decrease in 10 mg/kg/day and 10 mg/kg/day + stress.

The time-dependent effect of PB on AChE activity was determined in the final experiment. In this analysis, an increase was seen in AChE activity at 1 and 3 days post surgery in the sham controls. The activity at day 7 was closer to values seen in previous experiments. The results suggest a link between the surgery and higher AChE activity levels at 1 and 3 days for the sham operation. This link appeared to be attenuated by 7 days. This could indicate an increase in expression of AChE protein. At day 1, the AChE activity was inhibited by PB, suggesting that PB might enter the hypothalamus. However, at day 7, with lower AChE activity in sham controls, the addition of 10 mg/kg/day PB resulted in an increase in AChE activity. This suggests that the continued presence of PB caused an increase in AChE protein expression. We are currently running and analyzing Western blots to evaluate AChE protein levels in response to the PB treatment.

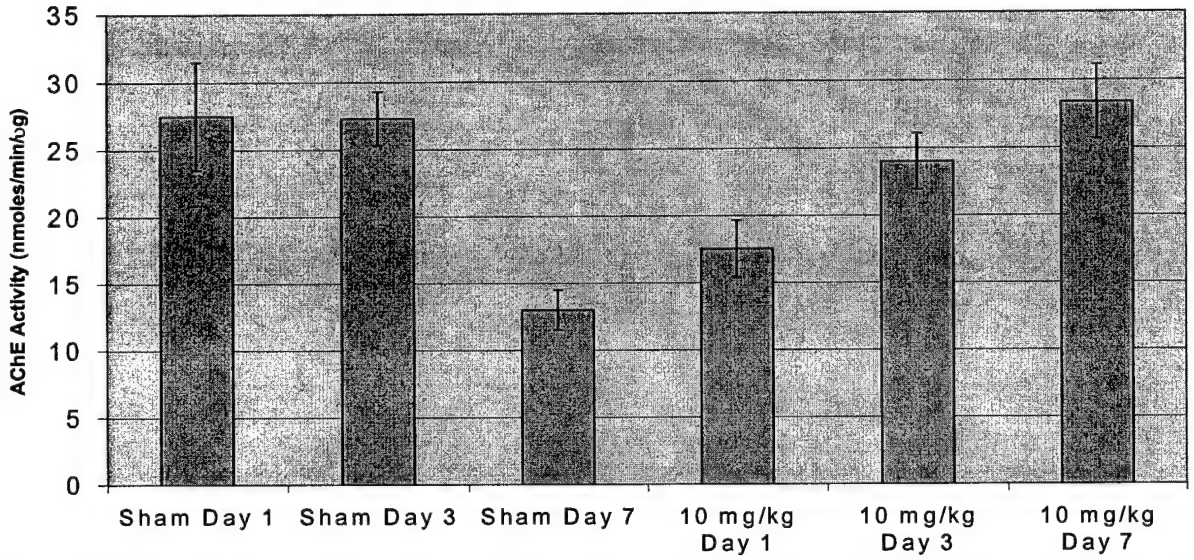


Figure 1-13. Summary of hypothalamic P02A experiment. The mini-pumps infusing saline (sham) or 10 mg/kg/day pyridostigmine Br were implanted for 1,3 and 7 days after which the animals were sacrificed and the hypothalamic AChE activity assayed.

2. Developed a method for Western blot analysis of AChE protein in hypothalamic tissue

To test the hypothalamic tissue for increased protein expression, 7 ug of the tissue was run on 10 % SDS-PAGE, transferred to PVDF membranes and probed with antibodies for AChE. The first Western blots showed several bands for AChE and we are currently using different antibody dilutions to more specifically analyze only the specific protein. Antibodies have been used to probe actin as a standard housekeeping protein in each lane of the blots, so that the loading can be normalized (Figure 1-14). The results presented, indicate a very faint band for 7 day sham. A slightly heavier band was observed for the 7 day- 10 mg/kg/day PB treated samples. The actin band is much heavier and will be used to normalize the protein levels in each lane.

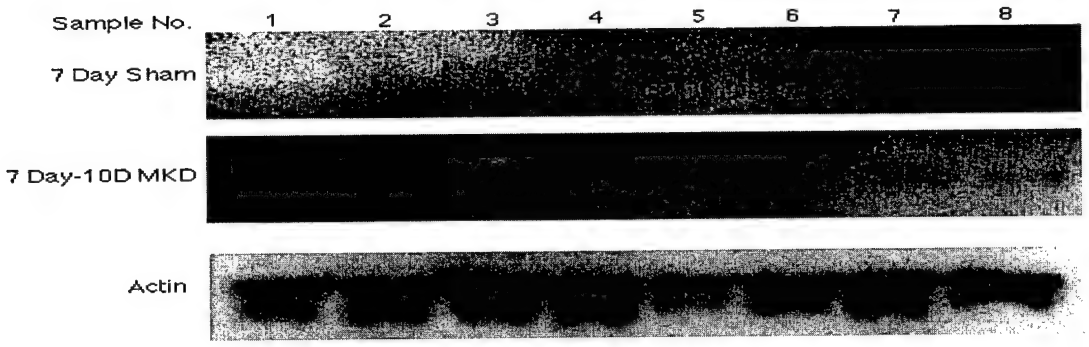


Figure 1-14. Western blot analysis of hypothalamic tissue from sham and 10 mg/kg/day infused mice. The polyclonal antibody recognized a band of approximately 72 kDa. The actin band is approximately 42 kDa.

3. Analyzed hypothalamic, anterior and posterior pituitary tissue using proteomic techniques

Pituitary and hypothalamic tissue was analyzed from each experiment using SELDI-TOF mass spectrometry to determine changes in the protein profiles. A summary of the preliminary analysis indicates that there is a dose-dependent decrease in some hypothalamic proteins as a result of treatment with pyridostigmine Br. However, changes were most apparent in the 3 and 10 mg/kg/day pyridostigmine Br treatment groups. Pituitary peptide expression was also affected, though some stress peptide expression was attenuated and other peptide expression was potentiated in the same treatment groups (Figure 1-15). The data provided from these experiments is currently being compared and analyzed and we expect to have a more thorough analysis by the next quarterly report.

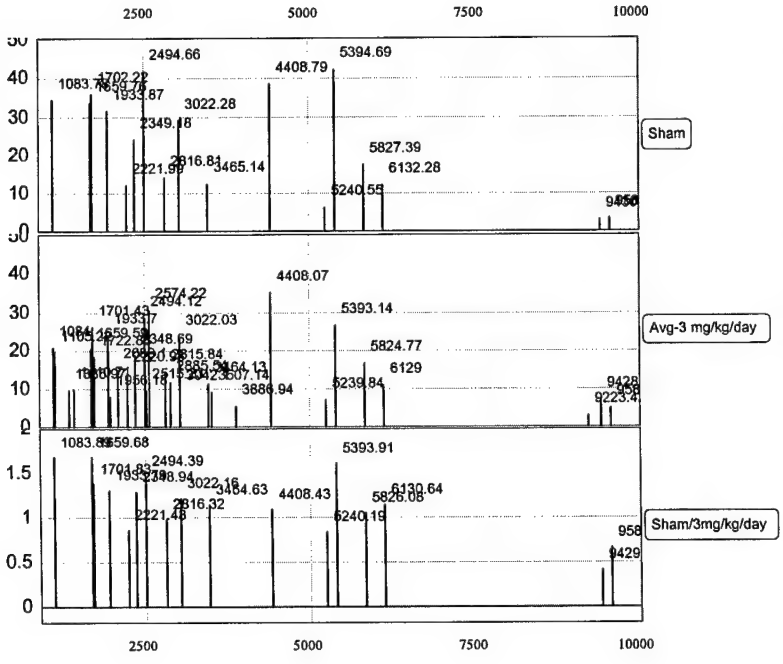


Figure 1-15. SELDI-TOF MS summary of posterior pituitary data from sham and 3 mg/kg/day pyridostigmine Br-treated mice. Top two panels represent the average intensities for each peptide peak (mass-m/z). The bottom panel represents the ratio of sham divided by the 3 mg/kg/day intensities. An intensity >1 indicates lower expression of a particular peptide and a peak of <1 indicates a higher expression compared to sham. 1= no

In an effort to determine the identity of peptides that were affected, control pituitary lysates were fractionated by size on an HPLC. Based on the HPLC retention times of standard proteins of similar size, specific fractions were analyzed by SELDI-TOF MS. One peak has been purified to homogeneity (9600 M_r) (Figure 1-16). Several other peaks have been purified to contain only one major peak (Fractions 22 and 23) and a minor peak (5861 M_r) (Figure 1-16). We are beginning tryptic digests and carboxypeptidase Y (C-terminal) digestive sequencing of these proteins in an effort to determine their identity. This data will provide us with more information about the effect PB and eventually sarin on peptide hormones of the HP-axis.

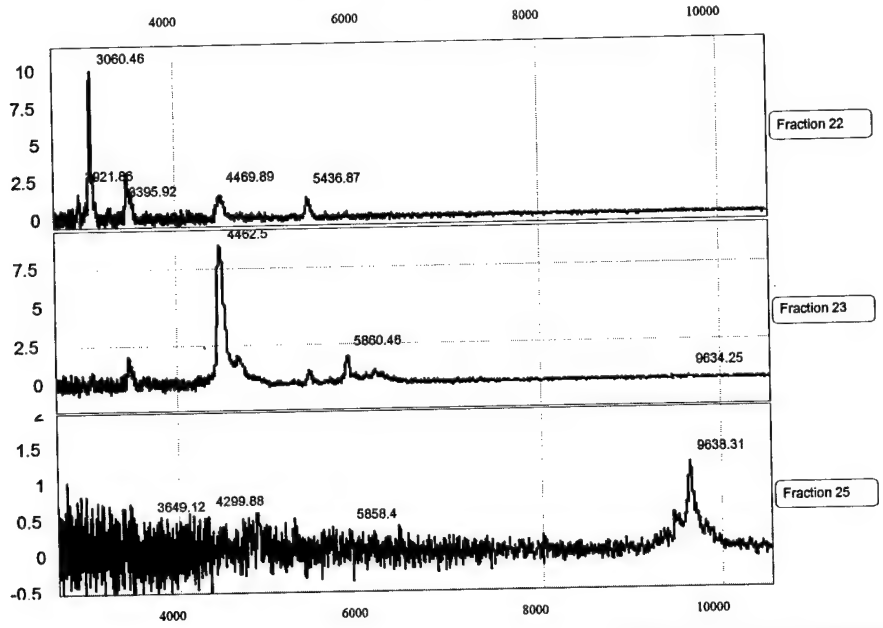


Figure 1-16. SELDI-TOF analysis of posterior pituitary peptides and proteins purified by HPLC size fractionation. Fraction numbers indicate the HPLC fraction collected (0.25 ml). The mass of the peptides is shown on the X-axis (m/z).

4. Developed a method for real-time PCR measurement of mRNA for AChE, GAPDH and hypothalamic peptides.

To completely understand the effect of PB on protein expression patterns, it is necessary to study the mRNA expression for the particular proteins of interest. For this reason, we developed the real time PCR method, which involved designing the primer sets (Table 1-2). The primers were designed to be approximately 20 base pairs long, have a melting temperature between 59 and 60°C and to have no dimers. The primers were designed using web-based software (Qiagen) and tested in a BioRad iCycler RealTime PCR Machine. A single PCR band was obtained for each primer set, indicating the ability of the PCR to accurately amplify only the mRNA of interest (Figure 1-17A). The PCR amplification fluorescence curves for each primer set are also shown in figure 1-17B. The curves show the relative amount of each mRNA species in the same hypothalamic sample. We will be using this technology to further study the effects of pyridostigmine Br, sarin and stress on peptides and enzymes of the HP-axis.

Table 1-2. Primer sets designed for RealTime PCR.

Primer	GC %	Tm	Product Size bp's	Dimers	Sequence- 5'---3'- Top- left primer/ Bottom- right primer
POMC-NM_008895	55	59.86	154	No	TAGATGTGTGGAGCTGGTGC
	50	60.23		No	TTTTCAGTCAGGGGCTGTTC
GAPDH M32599	50	59.89	110	No	AGAACATCATCCCTGCATCC
	55	60.09		No	CACATTGGGGGTAGGAACAC
Mouse c-fos V00727	55	59.69	112	No	GTCCGGTTCCTTCTATGCAG
	60	60.03		No	AGTACAGGTGACCACGGGAG
Mouse c-jun X12740	50	59.84	137	No	ATGGGCACATCACCACTACA
	55	60.31		No	GACACTGGGAAGCGTGTTCT
AChE X56518	50	59.98	101	No	GCAGCAATATGTGAGCCTGA
	45	60.18		No	TGAGCAATTTGGGGAGAAAG
Mouse AVP NM_00937	55	60.08	115	No	ACACTACGCTCTCCGCTTGT
	52	60.04		No	CACTGTCTCAGCTCCATGTCA

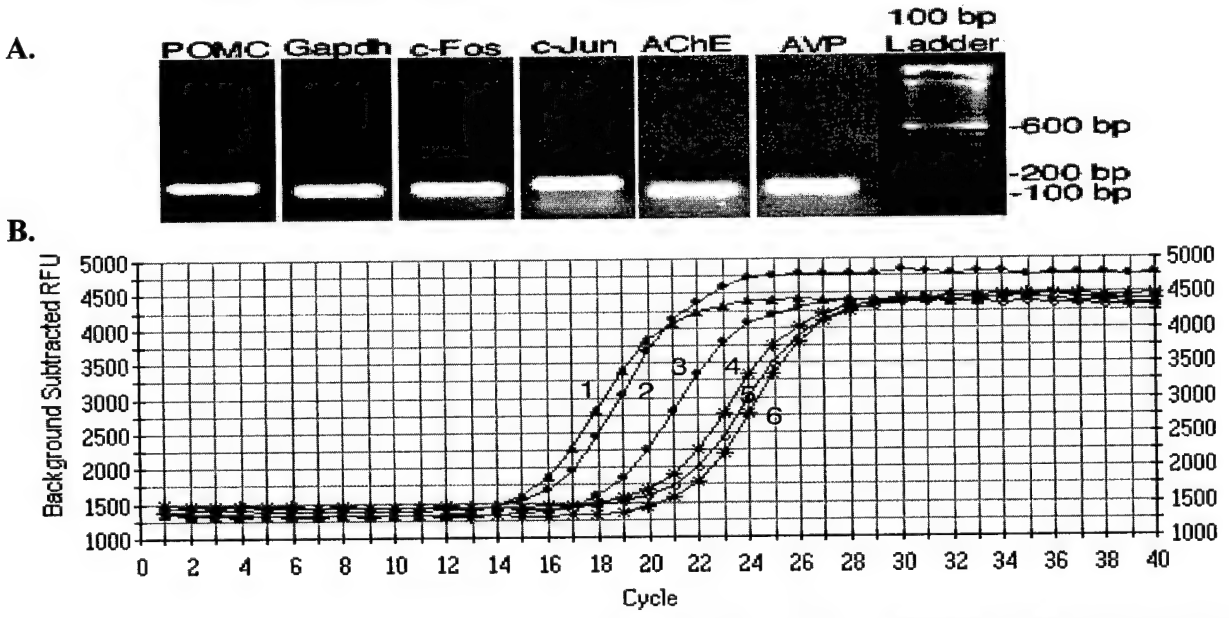


Figure 1-17. A) Agarose gel analysis of PCR products for POMC, GAPDH, c-fos, c-jun, AChE and AVP primer sets. Total RNA was prepared from hypothalamic tissue and 50 nanograms were used in each amplification reaction. B) Realtime PCR fluorescence curves showing the relative amounts of each mRNA in the hypothalamic tissue. The most abundant mRNA is on the left and the least abundant is on the right. The curves are, from left to right: 1) GAPDH, 2) mAVP, 3) AChE, 4) POMC, 5) c-jun and 6) c-fos.

Presentations and Publications:

Abstracts (See Appendix):

Ropp, S., Bernatova, I., Paton, S.J., Price, W.A., Morris, M. and Cool, D.R., SELDI-TOF mass spectrometry proteomic analysis of pyridostigmine Br and stress on the HP-axis. The Toxicologist 66: 1086 , 2002. Presented at the Poster-Discussion: "Toxicologic Application of Proteomics.

Publications:

Cool, D.R. and DeBrosse, D., Comparison of preparative methods for analysis of peptide hormones by radioimmunoassay and SELDI-TOF MS. In Revision, J. Chromatography B, 2002.

CARDIOVASCULAR/ENDOCRINE

Investigators Mariana Morris, Ph.D. and Iveta Bernatova, Ph.D.

The objective of this phase of investigation was to study the cardiovascular and endocrine effects of stress and chemical exposure. This project involves the 1) characterization of the stress model 2) determination of the effect of chronic PB treatment 3) determination of the effect of stress and PB combination 4) evaluation of the stress response in an animal model of altered stress reactivity, the oxytocin knock out mice

KEY ACCOMPLISHMENTS:

- Completed studies to evaluate the cardiovascular and endocrine changes associated with chronic stress in mice. Results suggest that chronic shaker stress is a viable model for stress exposure in mice.
- Evaluated the circadian pattern in cardiovascular and endocrine responses in mice. We found that there is a hyper-reactivity to stress during the day (inactive) period. This bears relevance to the timing of cardiovascular pathologies (heart attack and stroke) in humans.
- Setup the breeding colony for oxytocin gene deletion mice, including development of the PCR methods and establishment of a computerized data base.
- Initiated studies using a new genetic model, the oxytocin knock out. Preliminary results show that there are prominent changes in the stress responses after deletion of the oxytocin gene.
- Gave presentations at National Meetings (Society of Toxicology, Experimental Biology Meeting, Biosciences Review).
- Submitted one paper for publication, which describes the circadian variation in the cardiovascular stress response. Two other manuscripts are in preparation

1. Characterization of model of chronic shaker stress in mice (metabolic and endocrine studies)

A specialized laboratory was established for testing of cardiovascular parameters in catheterized mice exposed to stress. This laboratory includes computerized data acquisition system as well as specially designed caging system that is attached to a shaking device. The shaking device was programming to provide shaker stress over the period of 24h for 7 days. Caging system allows continual cardiovascular monitoring in undisturbed mice. This system was combined with other data collection system such as continual recording of drinking activity and pain-free collection of blood samples for investigation of endocrine function (corticosterone levels).

Initial studies were conducted in C57Bl mice. Characterization studies include determination of the stress effect on body weight, food intake, drinking activity and

corticosterone levels in chronic stress. Results showed that chronic stress decreased body weight about 5% after the first day of shaking (Fig 1-18) without significant changes in food intake (data not shown). BW differed during basal, stress and recovery periods ($F(2,107)=3.21$, $p<0.05$ for main effect of stress).

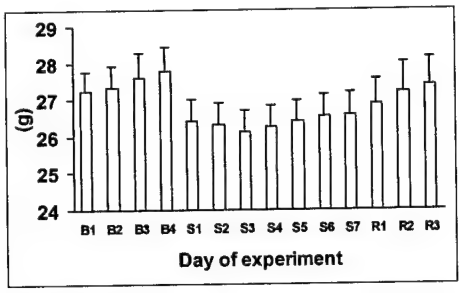


Fig 1-18

Figure 1-18. Effect of chronic shaker stress on body weight. B1-B4--days of basal recording; S1-S7--days of stress; R1-R3-- days of post-stress recovery.

Circadian data of licking activity were recorded using a computerized system with a sampling rate of 85 samples/sec. Stress did not alter the diurnal pattern of drinking (Fig 1-19 and 1-20) that was significantly concentrated in the dark period ($F(1,128)=345.27$, $p<0.0001$ for main effect of circadian factor). Stress significantly increased drinking activity of mice in the dark period ($F(1,128)=5.90$, $p<0.02$ for interaction of stress and circadian factor) as compared to non-stress periods (basal and recovery). On the basis of drinking activity, the analysis of the active (alpha) and non-active (rho) phase was performed. The beginning and end of the alpha phase corresponded with the beginning and end of the dark phase during all of the experiment. There were no differences in duration of the active alpha-phase and non-active rho-phase in stress vs. basal values (Fig 1-19). Thus, stress did not disrupt the diurnal rhythm of water intake even though there were experimentally imposed alterations in the sleep cycle.

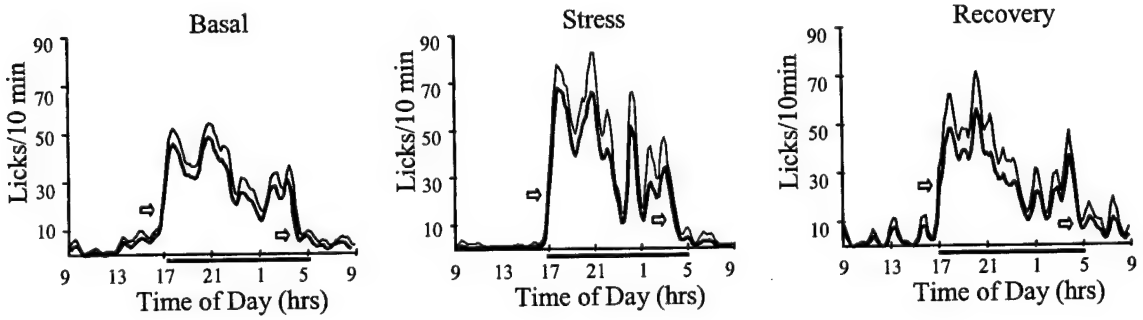


Fig. 1-19. Circadian pattern of drinking activity, comparing basal, the first day of stress and the third day of the recovery period. The arrows indicate the calculated onset and end of the active drinking period. The heavier part of the *x*-axis represents the dark period. Values represents average waveform (dark line) and average waveform positive error (light line).

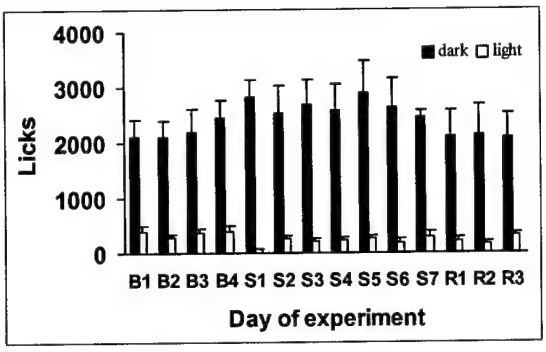


Figure 1-20. Effect of chronic stress on drinking activity. B1-B4--days of basal recording; S1-S7--days of stress; R1-R3--days of post-stress recovery. Stress increased significantly drinking activity of mice in the dark period ($F(1,128)=5.90$, $p<0.02$ for interaction of stress and circadian factor). Values are mean ± SEM.

Plasma corticosterone (Cort) levels, used as a marker of stress exposure, were compared in mice exposed to acute or chronic stress. Stress produced significant increases in plasma Cort under acute and chronic conditions ($F(2,65)=65.8$, $p<0.0001$), $p< 0.0001$ for main effect of stress). The acute response was about 2.5-fold greater than the chronic response ($p< 0.0001$). In chronically stressed mice, a 4-fold increase of Cort was found 30 and 60 min after stress as compared to the control levels ($p<0.01$). Long-term shaker stress increased plasma corticosterone levels, although the activation of the hypothalamic-adrenal (HPA) axis was partially attenuated as compared to the acute stimulus. The increases in plasma Cort after 7 days of stress suggests that shaker stress was an effective stimulus suitable for long-term studies.

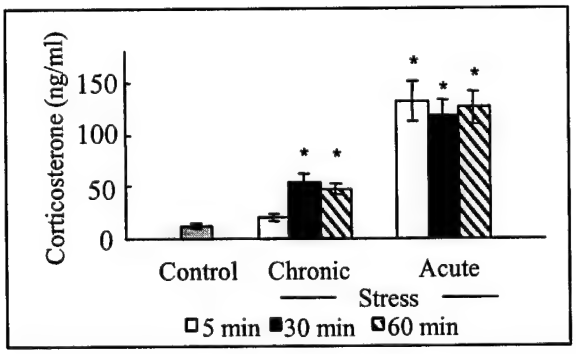


Figure 1-21. Plasma corticosterone response to acute and chronic shaker stress. Acute stress increased corticosterone concentrations significantly more as compared to chronic stress ($p<0.0001$). * $p< 0.05$ vs. control. Values are mean ± SEM

Characterization studies in Oxytocin Gene Deletion Mice:

Oxytocin is a key hypothalamic hormone in the stress cascade. The objective of this study was to examine the effect of chronic stress in male OT+/+ (control) and OT knockout (OT-/-, OTKO) age 3-6 months. The stress protocol used in this study was the same as used in

C57BL6 mice. There were no significant differences in body weight and food intake (Fig 1-22A and 1-22B). Interestingly, OTKO mice did not lose BW during stress, while in control mice (OT+/+) a similar decreasing trend of BW was observed.

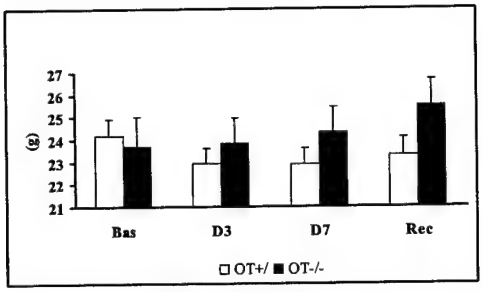


Fig 1-22A.

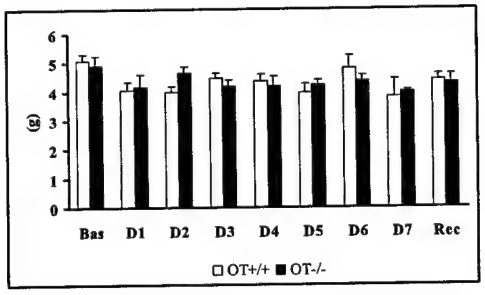


Fig 1-22B.

Figure 1-22. Effect of chronic stress on body weight and food intake in OTKO mice.

Drinking activity was measured by continuous (24h) recording of licking activity during basal, stress (days 1, 3 and 7) and recovery periods. Results were analyzed using 3-way ANOVA (genotype x treatment x day phase). Main effects of experimental day were significant ($F(4,114)=9.8$, $p<0.0001$) with decreased licking activity during stress period and recovery vs. basal values (in all mice regardless of genotype). There was a significant diurnal rhythm ($F(1,114)=651.1$, $p<0.0001$) with drinking concentrated during the dark phase. Time analysis within groups was done using 2-way ANOVA (day of experiment x day phase). There was a significant decrease in licking activity on day 1, 3, 7 and recovery ($p<0.01$) in the control group while in OTKO mice, licking was reduced only on days 1, 3 and 7 ($p<0.03$) vs. basal value (Fig 1-23A and 1-23B). These findings are different than those in C57BL mice where an increase of drinking activity was observed during stress.

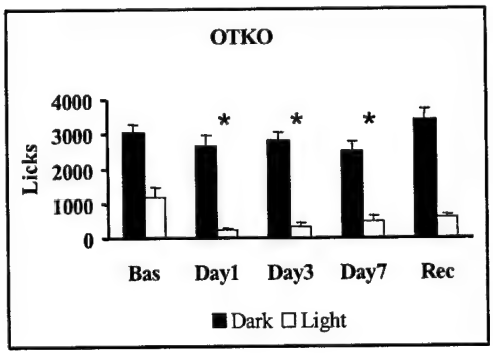


Fig 1-23A.

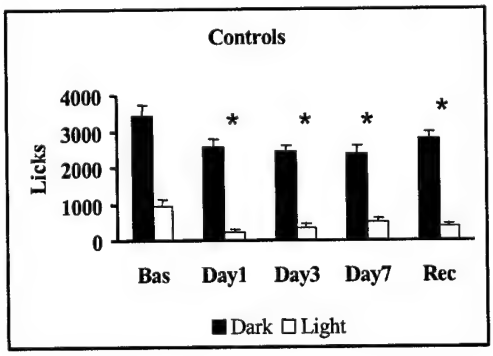


Fig 1-23B.

Figure 1-23. Drinking activity in OTKO and Control mice. *$p<0.03$ vs. basal values when 24-h averages were compared.

Stress induced differences in plasma Cort were evaluated using 2-way ANOVA (treatment x genotype) and Duncan's test. The main effect of treatment shows that stress produced significant increases in plasma corticosterone (Fig 1-24, $F(1,20)=26.0$, $p< 0.0001$). The main effect of genotype has shown a significantly higher level of corticosterone in control vs. OTKO mice ($F(1,20)=6.9$, $p<0.02$). The interaction of both factors has shown marginal significance ($F(1,20)=3.9$, $p=0.06$). Interestingly, the corticosterone levels in stress-exposed OTKO mice were reduced as compared to control. These data suggest that OTKO mice were less fearful and/or better adapted to stress exposure than control mice. However, behavioral tests are needed to understand these differences in OTKO mice.

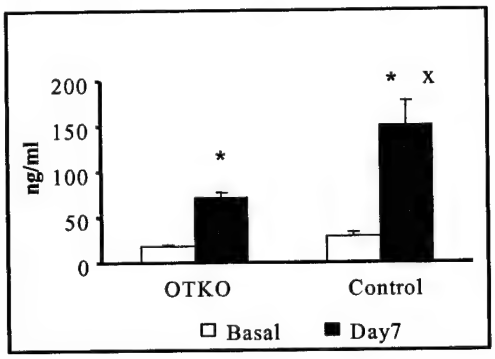


Figure 1-24. Effect of stress on corticosterone levels in control and OTKO mice. *$p<0.05$ vs. basal. $^{x}p<0.05$ vs. OTKO day 7.

B. Cardiovascular responses to shaker stress.

Mice were prepared with chronic carotid arterial catheters according to the method of Li et al. (Li P at al., *Am J Physiol* 1999; 276:R500-R504). After surgery, a heparinized saline solution (100U/ml) was continuously infused into the catheter at 25μl/h using a syringe pump (Model 220, Kd Scientific, Boston, MA). The catheter was covered with a metal spring that was attached to a fluid swivel at the top of the cage. The animals were allowed to recover from surgery for at least 5-6 days, by which time water and food intake had returned to normal. Blood pressure (BP) and heart rate (HR) were recorded continuously (24 h) on stress days 1, 3 and 7 and on the day before and after stress (basal and recovery). Differences in mean arterial pressure (MAP) and HR in the chronic experiment were evaluated by 2-way ANOVA (day of experiment x phase of day) followed by Duncan's test. Systolic BP, diastolic BP and HR were recorded with a sampling rate of 85 samples/sec and converted from digital to numeric form using acquisition software. Data were processed by calculation of 10-min means of the respective variable. These 10-min averages were averaged for calculation of the dark and light period means (Fig 1-25).

Initial studies were conducted in C57Bl mice. Stress responses of MAP and HR to individual shaking sessions were evaluated on stress days 1, 3 and 7 and on day 1 of the recovery period during the light and dark periods (1100 and 2300h). Responses were calculated as a percent of the values during the two minutes preceding the stress (Fig 1-26).

Stress responses of MAP and HR were recorded in the same manner as circadian data of MAP and HR, however data were processed by calculation of 2-min averages that were used for calculation of MAP and HR values before, during as well as after stress (divided to 1-8 min and 9-16 min post-stress). A statistical analysis was performed by 3-way ANOVA (day of experiment x phase of day x time course) and by Duncan's test.

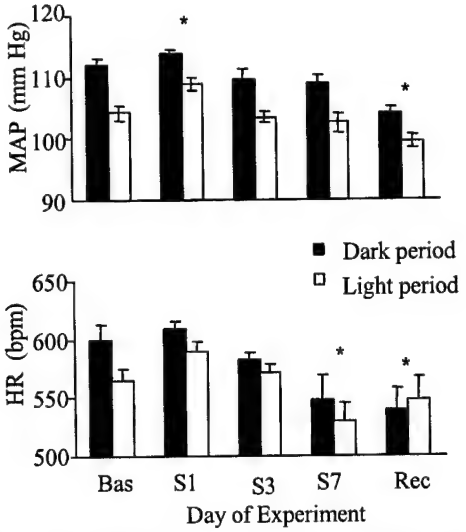


Figure 1-25. Mean arterial pressure (MAP) and heart rate (HR) of stress-exposed mice. Bas--before stress; S1, S3 and S7--days of stress; Rec--day 1 of the post-stress recovery. There were significant changes in overall (24h) MAP ($F(4,54)=17.93$, $p<0.001$; *$p< 0.008$ vs. day before stress) and HR ($F(4,54)=8.4$, $p< 0.0001$; *$p<0.003$ vs. day before stress). Values are mean ± SEM.

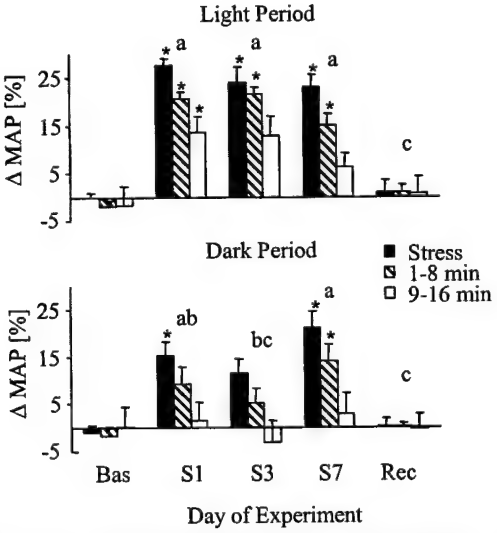


Figure 1-26. The time course of the stress-induced blood pressure changes on days 1, 3 and 7 of chronic stress. Response was measured during light and dark periods (1100 and 2300h) and calculated as a percent of the values during the two min preceding the stress. Results show MAP alterations during the stress (2 min) and after the stress (1-8 min and 9-16 min). [a]$p<0.01$ vs. basal day in the same period; [b]$p<0.001$ vs. light period on the same day; [c]$p<0.002$ vs. day 7 during the same period where a, b and c represent a comparison of overall response (i.e. average of stress,

1-8 min and 9-16 min). *$p<0.05$ vs. time-related values on the basal day. Values are mean ± SEM.

The analysis of the circadian pattern of stress responsiveness revealed some interesting findings. There was a marked increase in the pressor response when the stress was delivered during the light phase (non-active, sleeping period). In fact, on stress day 3, there were no significant changes in BP during the night (active period), but a 24% increase during the light. An examination of the behavioral activity associated with chronic stress showed a similar circadian pattern (see behavioral studies). Stress significantly decreased locomotor activity only during the day. The reason for the difference in the day/night cardiovascular and behavioral responses is not known but may be related to sensory activation (waking from sleep), changes in regional hemodynamics, input from circadian pacemakers, or other factors.

Oxytocin gene deletion:

Circadian data of systolic BP, diastolic BP and HR were recorded using the same method as in C57BL mice. Between group differences of MAP and HR were analyzed using 3-way ANOVA (genotype x day phase x day of experiment). The effect of stress within the groups was analyzed using a 2-way ANOVA (treatment x day phase).

Basal MAP in the control group was 111±2 and 104±3 mm Hg during dark and light periods (12-hr averages), respectively (Fig 1-27). In the OTKO mice, basal MAP was 106±2 and 100±1 mm Hg during dark and light periods (12-hr averages), respectively. The MAP of OTKO mouse was significantly lower than in the control group ($F(1,102)=24.55$, $p<0.0001$ for main effect of genotype). There was a significant diurnal rhythm of MAP ($F(1,102)=98.2$, $p<0.0001$ for main effect of circadian factor) in both experimental groups. Stress significantly increased MAP (24-h average) on day 1 vs. basal values in all animals regardless of the genotype ($F(4,102)=4.97$, $p<0.001$ for main effect of treatment). A within group analysis has shown the significant increase of MAP (24 hr average) in OTKO mice on the 1st and 3rd day of stress ($p<0.03$), which was not found in the control group (Fig 1-27).

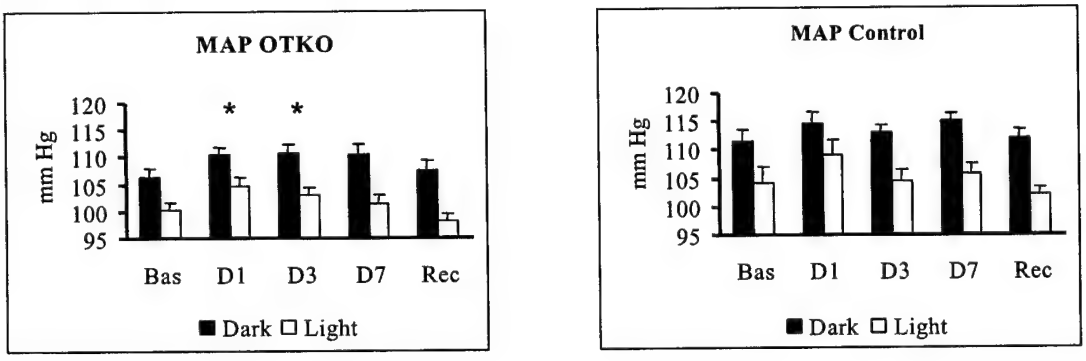


Figure 1-27. Mean arterial pressure (MAP) of stress-exposed OTKO and control mice. Bas--before stress; S1, S3 and S7--days of stress; Rec -- day 1 of the post-stress recovery. *$p< 0.03$ vs. day basal). Values are mean ± SEM.

Basal HR of control mice was 595±9 and 563±6 beats/min during dark and light periods (12-h averages), respectively (Fig 1-28). In the OTKO mice, basal HR was 581±7 and 548±12 beats/min during dark and light periods (12-hr averages), respectively. Heart rate of OTKO mice was significantly lower than in the control group ($F(1,92)=7.9$, $p<0.005$ for main effect of genotype). There was a significant diurnal rhythm of HR ($F(1,92)=46.24$, $p<0.0001$ for main effect of day phase) in both experimental groups. Stress significantly decreased HR (24-h average) on days 3 and 7 of stress and the recovery vs. basal values in all animals regardless of the genotype ($F(4,92)=7.72$, $p<0.0001$ for main effect of treatment). A within the group analysis has found no differences between HR (24-h average) of control and OTKO mice. A significant decrease of HR was found in both groups after cessation of stress.

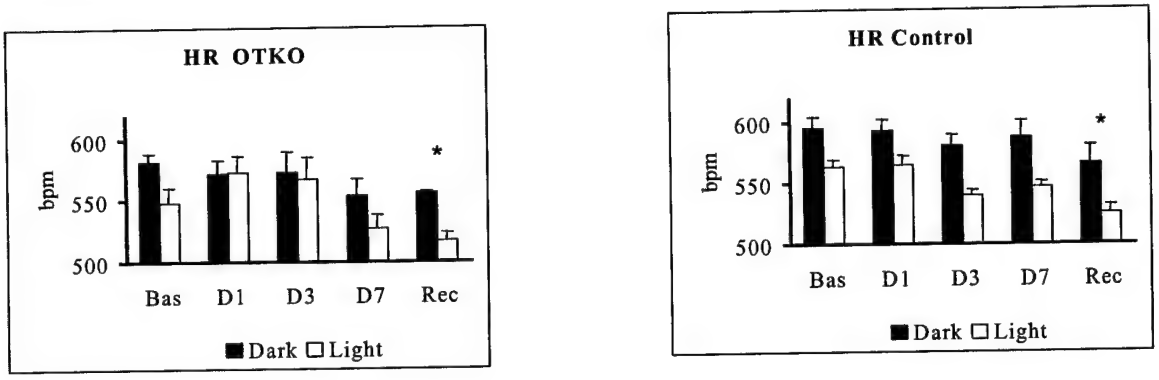


Figure 1-28. Heart rate (HR) of stress-exposed OTKO and control mice. Bas--before stress; S1, S3 and S7--days of stress; Rec--day 1 of the post-stress recovery. *$p<0.05$ vs. day before stress). Values are mean ± SEM.

In order to evaluate cardiovascular reactivity, we examined the pressor and heart rate responses during the light/dark periods on days 1, 3 and 7 of chronic stress. Between group analysis was performed using 4-way ANOVA (genotype x treatment x day phase x time course). Within group analysis was performed by 3-way ANOVA (treatment x day phase x time course).

The results showed that MAP of OTKO mice were lower than MAP of control mice ($F(1,352)=26.6$, $p<0.0001$). There was a diurnal pattern in MAP ($F(1,352)=129$, $p<0.0001$; Fig 1-29) with increased MAP during the dark period. The main effect of treatment showed significant differences in MAP ($F(4,352)=15.45$, $p<0.0001$) with significant elevation of MAP on stress days 1, 3 and 7 vs. basal value in all animals regardless of genotype.

The immediate increases of MAP in OTKO mice during stress in the light period were approximately 28%, 31% and 24% ($p<0.001$) on days 1, 3 and 7, respectively as compared to MAP 2-min before stress. The immediate increase of MAP in OTKO during stress in the dark period was 20% ($p<0.001$) on day 1 and non-significant alterations were found on day 3 and 7. In control mice, the immediate increase of MAP during stress in the light period was 23%, 24% and 26% ($p<0.001$) as compared to MAP 2-min before stress. Any significant differences were found in the dark period (Fig 1-29).

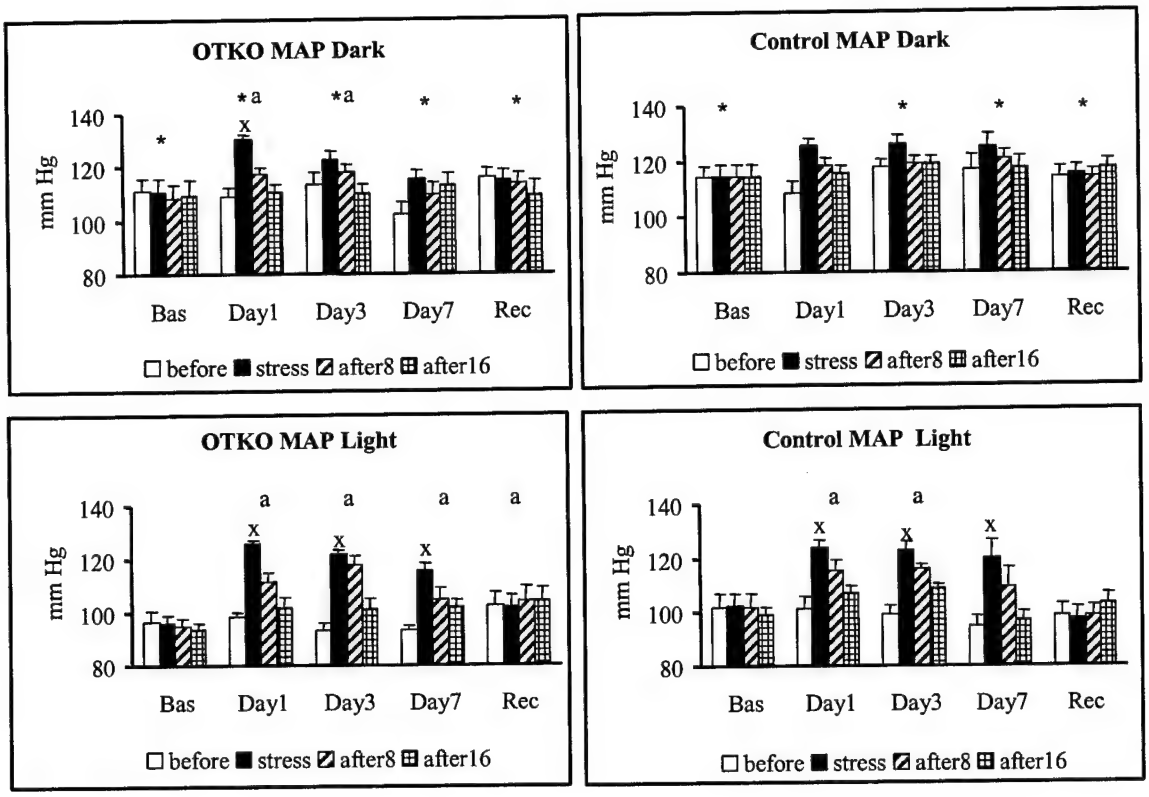


Figure 1-29. The time course of the stress-induced MAP changes on days 1, 3 and 7 of chronic stress in OTKO and control mice. Responses were measured during the light and dark periods (0800 and 1900h). Results show MAP alterations 2 min before stress, during the stress (2 min), and after the stress (1-8 min and 9-16 min post stress). *$p<0.05$ vs. light period on the same day; $^{a}p<0.05$ vs. basal value in the same period where * and "a" represent a comparison of overall response (i.e. average of MAP before stress, stress, 1-8 min and 9-16 min); $^{x}p<0.05$ vs. MAP before stress on the same day and period. Values are mean ± SEM.

Heart rate of OTKO mice was lower than that of control mice ($F(1,352)=13.5$, $p<0.0003$, for main effect of genotype). There was a diurnal pattern in HR ($F(1,352)=183.1$, $p<0.0001$ for main effect of day phase) with increased values of HR during the dark period (Fig 1-29). There were no differences in HR during stress exposure in any genotype of mouse (main effect of treatment).

The immediate increases of HR in OTKO mice during stress in the light period were approximately 38%, 36% ($p<0.02$) and 22% (NS) on days 1, 3 and 7, respectively as compared to MAP 2-min before stress. Only non-significant differences were found in the dark period. In control mice, the immediate increase of MAP during stress in the light period was 22%, 22% and 29% ($p<0.03$) as compared to HR 2-min before stress. All significant differences were found in the dark period (Fig 1-30).

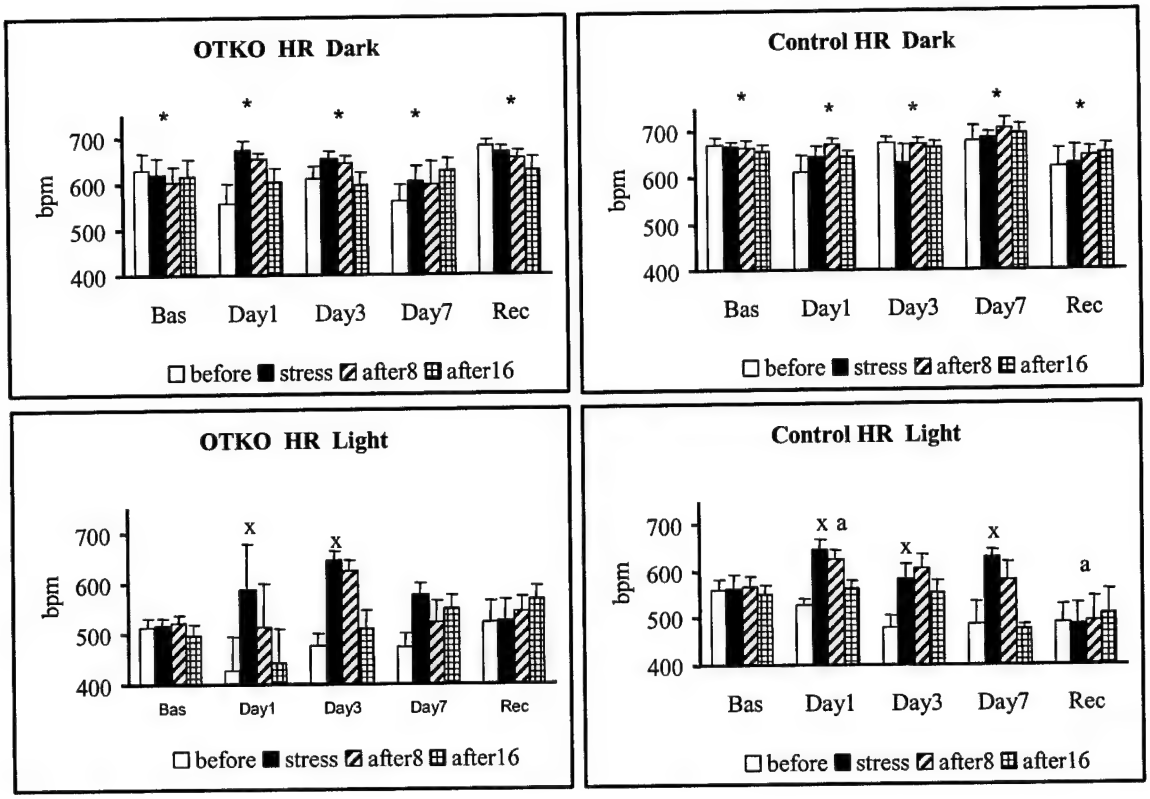


Figure 1-30. The time course of the stress-induced HR changes on days 1, 3 and 7 of chronic stress in OTKO and control mice. Responses were measured during the light and dark periods (0800 and 1900h). Results show HR differences 2 min before stress, during the stress (2 min) and after the stress (1-8 min and 9-16 min post stress). *$p<0.05$ vs. light period on the same day; $^{a}p<0.05$ vs. basal value in the same period where * and "a" represent a comparison of overall response (i.e. average of HR before stress, stress, 1-8 min and 9-16 min); $^{x}p<0.03$ vs. HR before stress on the same day and period. Values are mean ± SEM.

In conclusion, we developed and characterized a model for chronic stress exposure in mice that allows for the determination of BP and HR in the undisturbed state. The results showed that stress produced an activation of the HPA axis that was partially attenuated in the chronic condition. There was no sustained increase of MAP, but there were important circadian changes in pressor responsiveness. The study provides evidence that stress delivered during the non-active phase of day represents a higher cardiovascular risk than stress delivered during the active phase. This may have implications for the human condition in which there are circadian patterns in the incidence of cardiovascular problems as well as links between stress and heart disease.

Presentations and Publications:
The activities of this year focused on preparation of posters for the Society of Toxicology meeting in Nashville (1), Experimental Biology Meeting in New Orleans (2) and Bioscience Meeting in Maryland (3). Full abstracts are in the Appendix.

Abstracts:

Bernatova I, Price WA, Grubbs RD, Morris M. Effect of chronic pyridostigmine bromide treatment on blood pressure and acetylcholinesterase activity in mice. The Toxicologist 66: 2002.

Bernatova I, Dubovicky M, Key M, Lucot JB, Morris M. Chronic stress alters cardiovascular and endocrine responses in mice. The FASEB J. 16: A506, 2002.

Bernatova I, Dubovicky M, Paton SJ, Lucot JB, Morris M. Pyridostigmine treatment alters stress responsiveness in mice. Bioscience Review, Abstract Program Book, pp123, 2002.

Publications:

Bernatova I, Key M, Lucot JB, Morris M. Circadian differences in stress-induced pressor reactivity in mice. Hypertension, in revision, 2002.

Bernatova, I. M. Dubovicky, J. B. Lucot, R. Grubbs, W. Price, M. Morris: Effect of pyridostigmine treatment on behavioral and cardiovascular parameters in mice. in preparation for Pharmacology, Biochemistry and Behavior, 2002.

Project 2: The effect of stress and chemical exposure on auditory brain responses, energy metabolism and tissue chemical constituents in an animal model.

PIs: Ina Bicknell, Nicholas V. Reo, and Lawrence J. Prochaska; Department of Biochemistry & Molecular Biology, Wright State University

Project 2 was formerly denoted as Modules 2, 3, and 4 in the contract. Funding for this project began on 01-Oct-2000. Because the research activities in these three modules are closely interrelated, the research efforts of Project 2 are summarized in one report. This report concerns the activities for the period 01-July-2001 to 30-June-2002.

The PIs, technicians and students involved in Project 2 meet on a biweekly basis to plan and coordinate experiments, discuss data, and review the current literature in the field. A summary of the overall accomplishments is given below. This is followed by a detailed Progress Report that is organized into three sections: Section A concerns the ABR studies (PI: I. Bicknell), Section B concerns the NMR studies (PI: N.V. Reo), and Section C concerns the studies of mitochondrial energy metabolism (PI: L.J. Prochaska).

Project 2, Section A: Auditory brainstem response (ABR) studies (PI: Ina Rea Bicknell)

Introduction/Materials and Methods

Details of this repeated measures protocol for the study of the effects of chronic low doses of DEET, pyridostigmine bromide (PB), alone and in combination, and the effects of noise stress have been discussed in previous reports. Briefly, eighty- to one-hundred-day-old male Sprague Dawley rats were treated once weekly for 4 consecutive weeks with toxin; controls were administered an equal volume of vehicle. DEET (225 mg/kg in arachis oil) was given by intraperitoneal (ip) injection. PB (5mg/kg in saline) was given by intragastric gavage. For the study of the synergistic effects of toxins, DEET was administered 10 min before PB. Immediately after the weekly administration of toxin or vehicle, the animals were exposed to 8h of 85dBSPL (re: 20μPa) white noise. Animals were housed individually in cages during noise exposure.

ABR measurements were made 24 hours prior to the administration of the first dose of toxin or vehicle and noise exposure. Repeated measurements were made at 24 hours after the first toxin or vehicle administration/noise exposure and at 6 days after the first, and the following three, weekly toxin or vehicle administrations/noise exposures. The ABR waveform consists of a series of peaks and valleys; Peak II is a putative indicator of neural activity primarily in the brainstem cochlear nucleus, which receives input from the auditory nerve (Church and Overbeck, 1990). Peak II is the strongest peak in the rat ABR, and, because it is the last to disappear with decreasing intensity of the auditory signal, it is used for threshold evaluation. Thresholds were obtained using a modified method of limits. The acoustic signals were presented in an intensity series, beginning at 90 dBSPL and decreasing in 10-dB steps until near threshold, at which point the decrements were 3dB steps. Threshold was defined as the lowest stimulus intensity that elicits a reliably identifiable ABR response. Threshold evaluations are made independently by two judges.

Four acoustic signals were used to collect data: 3-ms tone bursts with frequencies of either 32-, 4-, or 8-kHz and alternating condensation/rarefaction clicks having a duration of 100-μs. Clicks were presented at a rate of 23/s. Auditory stimuli were presented binaurally via pediatric ear inserts.

Statistical analyses are currently being done in consultation with the Statistical Consulting Center at Wright State University. The data are being evaluated by a repeated measures ANOVA with ABR Peak II thresholds, latencies, and amplitudes as the measures repeated over time. Groups (test and control) and absence or presence of stress are between variables; time is the within variable. Data from the studies of the individual toxins, DEET or PB, have been collected; the Statistical Center has formatted the data and is completing the analysis. Data from the synergistic study of DEET plus PB will be analyzed upon completion of the testing of Group II animals and the repetition testing of Group I (repetition discussed in a later section).

Results
Toxins without and with noise stress

DEET without and with noise stress

Peak II thresholds

The effects of DEET, without and with noise stress, on the threshold of Peak II of the ABR waveform are shown in Figures 2-1 and 2-2, respectively. No obvious differences in ABR Peak II threshold with time, either without or with noise stress, were observed for any of the four auditory signals employed. The numerical differences in threshold that were associated with specific acoustic signals reflect the differential sensitivity of the basilar membrane hair cells in the rat cochlea. These frequency-specific threshold differences, therefore, should not be interpreted as a consequence of the treatment.

Control vs. DEET and absence of noise vs. exposure to noise thresholds were very stable for the click stimulus. The click is the most robust signal, a consequence of widespread synchronous neural activity along the basilar membrane induced by the broad frequency content and the sharp onset of the acoustic signal. Even in the absence of statistical analysis, it is clear there were no differences among the conditions for this acoustic signal. Until the statistical analysis is complete, we cannot say if the small differences in Peak II threshold observed for the 8-kHz and 4-kHz signals across time are significant (Figs. 2-1 and 2-2, Panels B and C). In a few instances, thresholds of the toxin-treated rats compared to the vehicle-treated animals varied by as much as 4-7 dB. For example, the 32k-Hz threshold for the control animals at Week 2 was 7 dB higher than that of the DEET-treated rats (Fig. 2-1, Panel A). Thresholds obtained from animals exposed to noise (Fig. 2-2, Panels A-C) tended to somewhat higher than those of the non-exposed rats (Fig.2-1, Panels A-C) for the frequency-specific signals. Again, this difference is so small that statistical analysis will be needed to determine whether this difference is meaningful.

Latency/Amplitude

In addition to threshold effects, two other Peak II parameters of the ABR waveform were examined for evidence of DEET toxicity and noise stress effects. The latency of Peak II is indicative of conduction time along the neural fibers and intervening synaptic delays (Katz et al., 2002) and was measured as time from the arrival of the stimulus at the rat's ears to the vertex of Peak II. Amplitude reflects the amount of neural activity and/or the degree of synchronization (Katz et al., 2002) and was measured as the distance from the vertex of Peak II to the following trough in the ABR waveform. Because of time constraints, the latencies and amplitudes were calculated at 90 and 50 dBSPL only. These two levels were chosen simply because 90 dB was the highest intensity at which the acoustic signals were presented, and 50 dB was at the approximate mid-point of the intensity series. Because there were no obviously significant effects of toxin or noise on the thresholds for any of the acoustic signals, latencies and amplitudes of 32-kHz waveforms only were analyzed. The frequency of this signal falls within the most sensitive portion of the audibility curve for Sprague Dawley rats (Kelley and

Masterson, 1977). Figure 2-3 shows repeated measures of 32-kHz Peak II latency at 90 and 50 dB of DEET-treated and control rats, in the

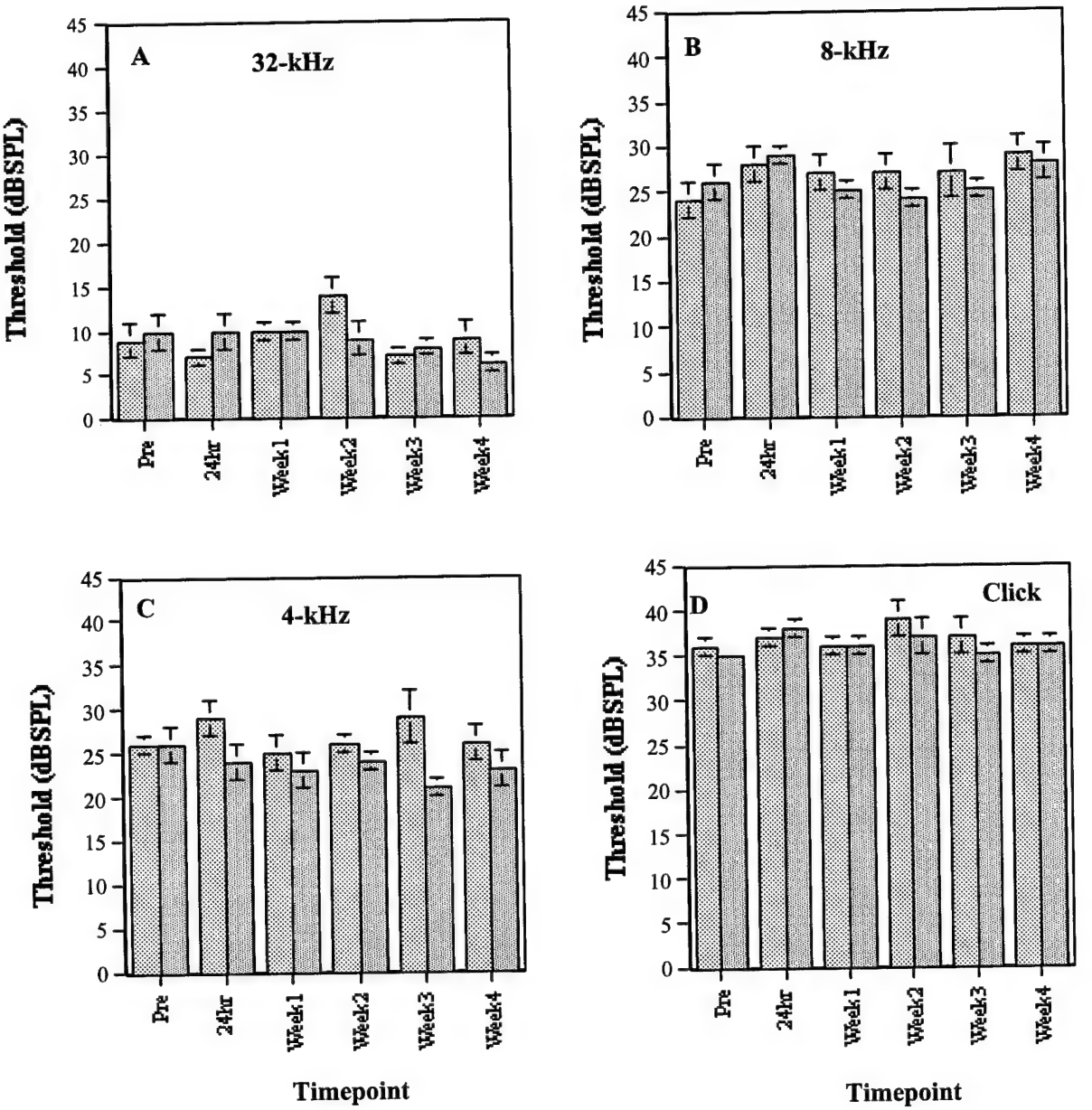


Figure 2-1: DEET without noise stress: Peak II thresholds. Experimental animals administered 225mg/kg DEET (ip) weekly for four weeks. Control animals injected with equal volume of arachis oil (ip). Thresholds are expressed as the mean ±SEM from either 6 (controls) or 8 (exp) independent observations (N). A: 32-kHz stimulus; B: 8-kHz stimulus; C: 4-kHz stimulus; D: Click. Timepoints: 24 h before the DEET or oil (pre); 24 h after the DEET or oil (24h); one to four weeks after exposure to first dose of DEET or oil (Week1-Week4). Legend: ▨ CONTROL ▨ EXPERIMENTAL

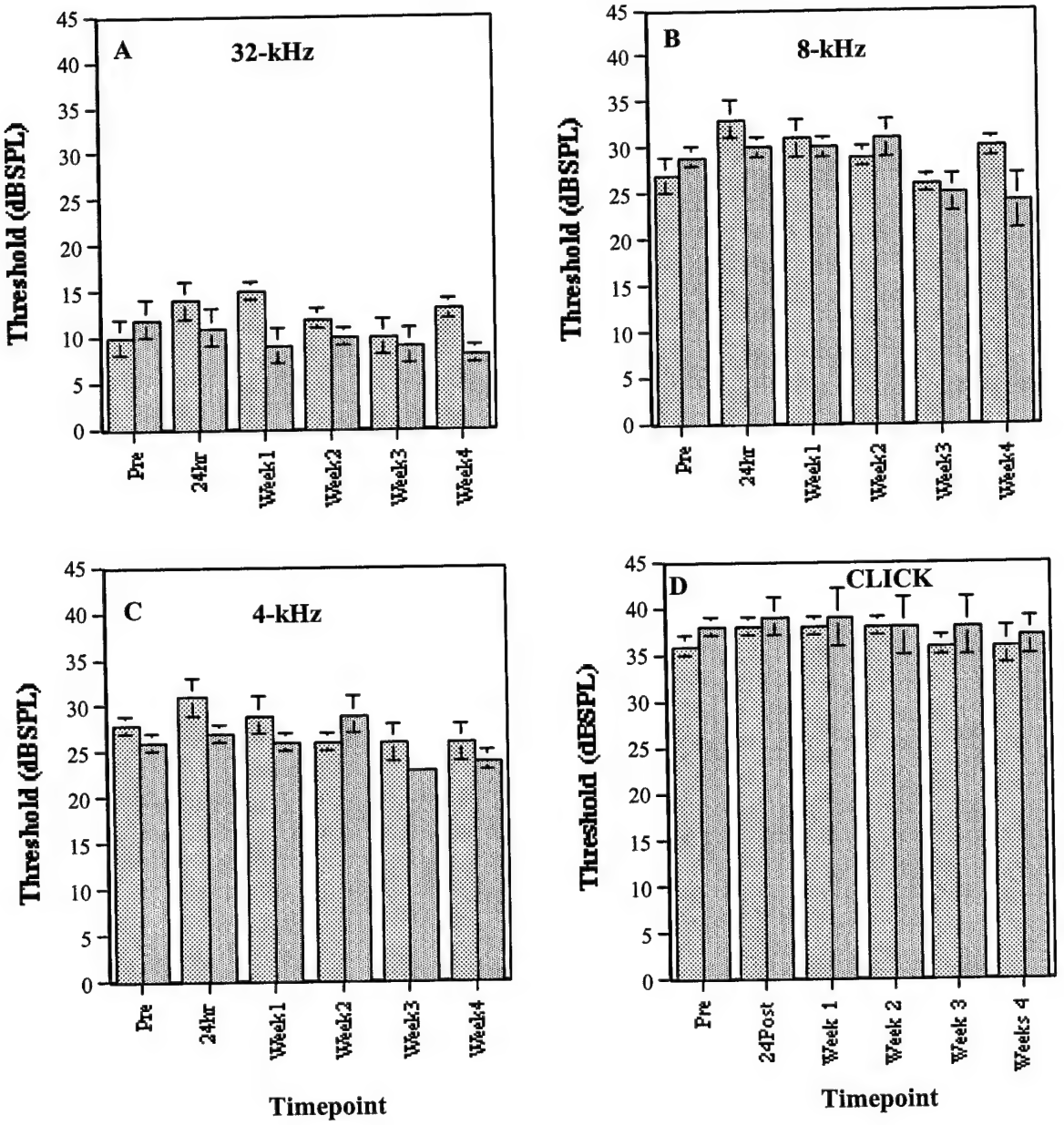


Figure 2-2: DEET plus noise stress : Peak II thresholds. Experimental animals administered 225mg/kg DEET (ip) weekly for four weeks. Control animals injected with equal volume of arachis oil (ip). Immediately after DEET or oil administration, animals were exposed to 8h of 85dBSPL white noise. Thresholds are expressed as the mean ±SEM from either 6 (controls) or 8 (exp) independent observations (N). A: 32-kHz stimulus; B: 8-kHz stimulus; C: 4-kHz stimulus; D: Click. Timepoints: 24 h before DEET or oil (pre); 24 h after DEET or oil (24h); one to four weeks after exposure to first dose of DEET or oil (Week1-Week4). Legend: CONTROL DEET

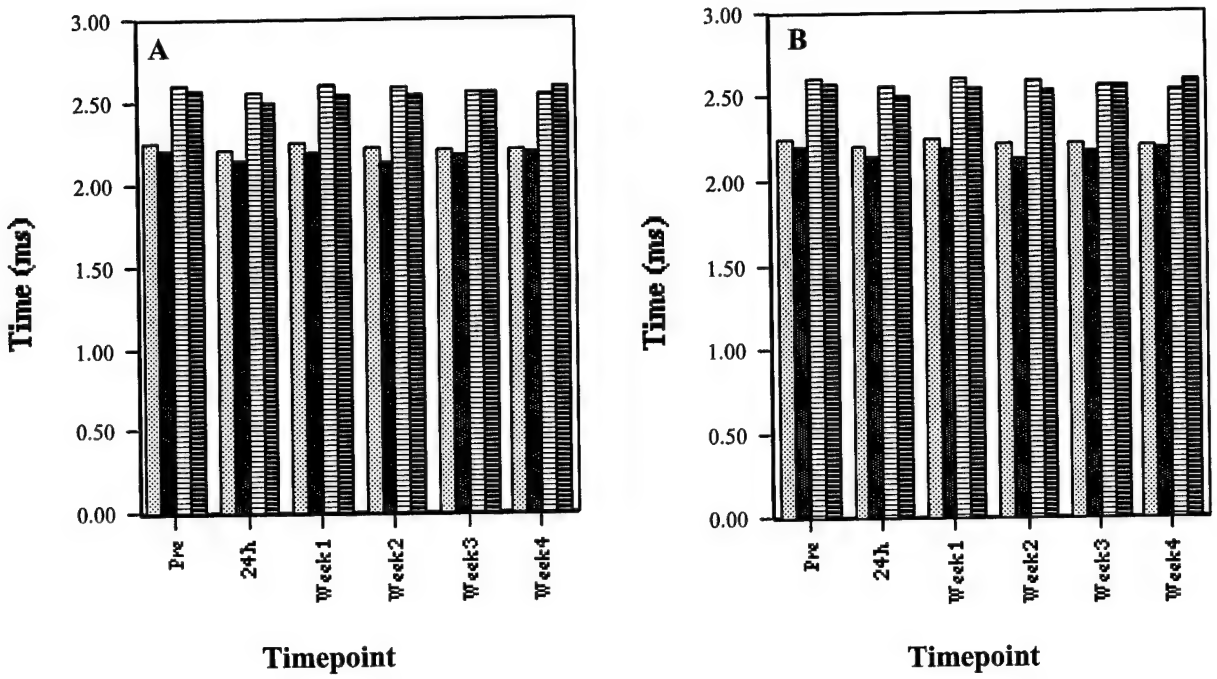


Figure 2-3. Effect of DEET ± noise on 32-kHz Peak II latency at 90 and 50 dB. A: No noise; B: With 8h of 85dBSPL white noise. Values are expressed as the mean from either 6 (controls) or 8 (experimental) independent observations. All SEMs<0.1ms. Legend: DEET 90 CONTROL90 DEET 50 CONTROL50

absence (Panel A) and presence (Panel B) of noise stress. As anticipated, the 50-dB latencies were longer than the 90-dB latencies. There were no statistically significant differences between the DEET-injected animals and the vehicle-injected controls, either in the absence (Panel A) or presence (Panel B) of noise stress. A comparison of thresholds in Panel A to those in Panel B shows that noise stress had no effect on Peak II latencies in either the toxin-injected or the control animals

Peak II amplitudes generated by the 32-kHz signal were higher at 90 dB than at 50 dB, as expected (Figure 2-4). Peak amplitude is a highly variable parameter (Katz et al., 2002); therefore, the observed differences between the Peak II amplitudes of ABR waveforms from DEET-injected and vehicle-injected animals are probably not statistically significant, either in the absence (Panel A) or presence of noise stress (Panel B). Because of the high degree of variability in the data, it is difficult to determine, without the statistical analysis, if the amplitudes in the noise exposure condition are significantly lower (compare Panel A to B). The intriguing observation in Figure 2-4 is that the 50-dB amplitudes of the DEET-injected and the control animals are the same under no-noise conditions, but the post-injection 50-dB amplitudes of the DEET-injected rats tend to be higher than those of the control animals. Again, one cannot say, yet, if the differences are significant.

PB with noise stress

Peak II thresholds

Chronic administration of PB and exposure to noise stress had no obvious effect, across time, on Peak II thresholds for any of the acoustic signals (Figure 2-5). The mean threshold intensities were nearly the same for both PB-administered rats and vehicle-treated controls across the four-week study.

Latency/amplitude

As can be seen from Figure 2-6, no toxic effects on the latency/intensity function were observed for the 32-kHz signals across time. The latency/intensity functions for all timepoints (pre, 24h, 1-4 weeks) for both toxin- and vehicle-treated animals are nearly superimposable. As noted earlier, latency is a stable parameter of ABR waveform peaks (Katz et al., 2002); in this study, the SEMs for the mean latency values from the six timepoints, for both PB- and vehicle-administered animals, was less than ±0.1 ms.

The amplitude/intensity functions were fairly similar in shape and slope (Figure 2-7). Although Peak II amplitudes were highly variable for both PB- (Panel A) and vehicle-treated animals (Panel B), the mean amplitude values for both groups fell within the range of 1μV to 3.4 μV. The absence of unusually large increases in amplitude with increasing intensity in any of the PB plus noise stress curves indicates that the toxin did not induce loudness recruitment. Surprisingly, Peak II amplitudes tended to be lower at 90 dB than at 80 dB. This decrease may a consequence of reflective characteristics of the ultrasonic signal (32 kHz) within the ear canal at high intensities.

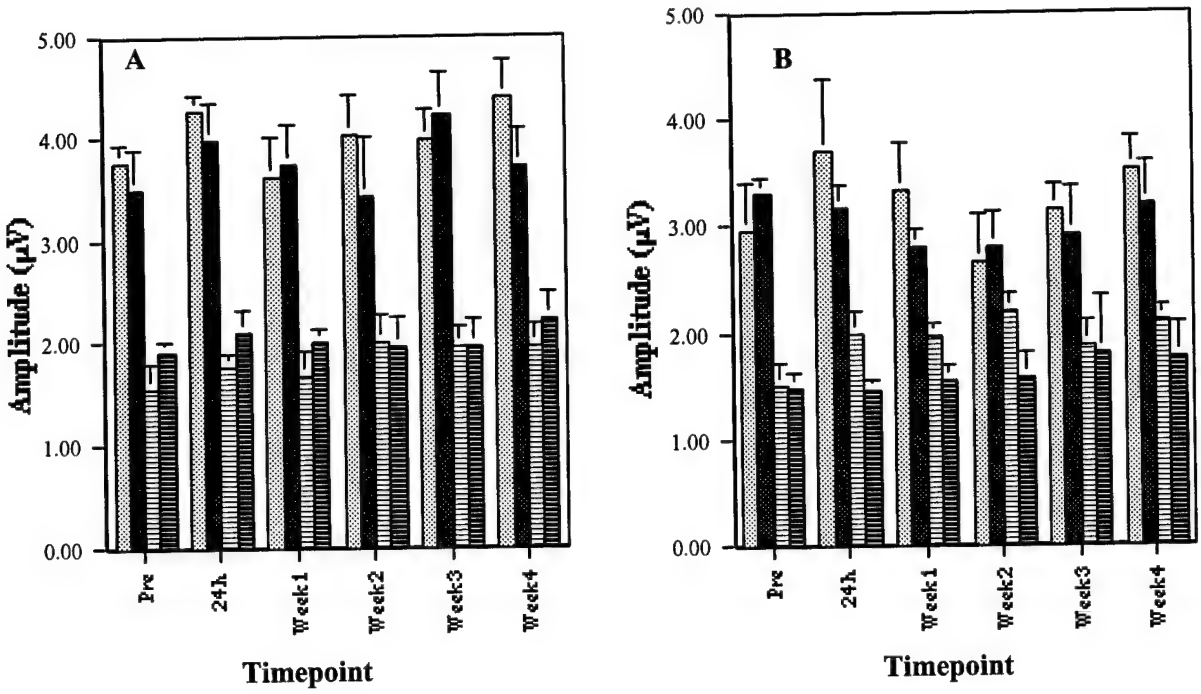


Figure 2-4. Effect of DEET ± noise on 32-kHz Peak II amplitude at 90 and 50 dB. A: No noise; B: With 8h of 85dBSPL white noise. Values are expressed as the mean from either 6 (controls) or 8 (experimental) independent observations. Error bars represent +SEM (equal -SEM not shown). Legend: DEET 90 CONTROL90 DEET 50 CONTROL50

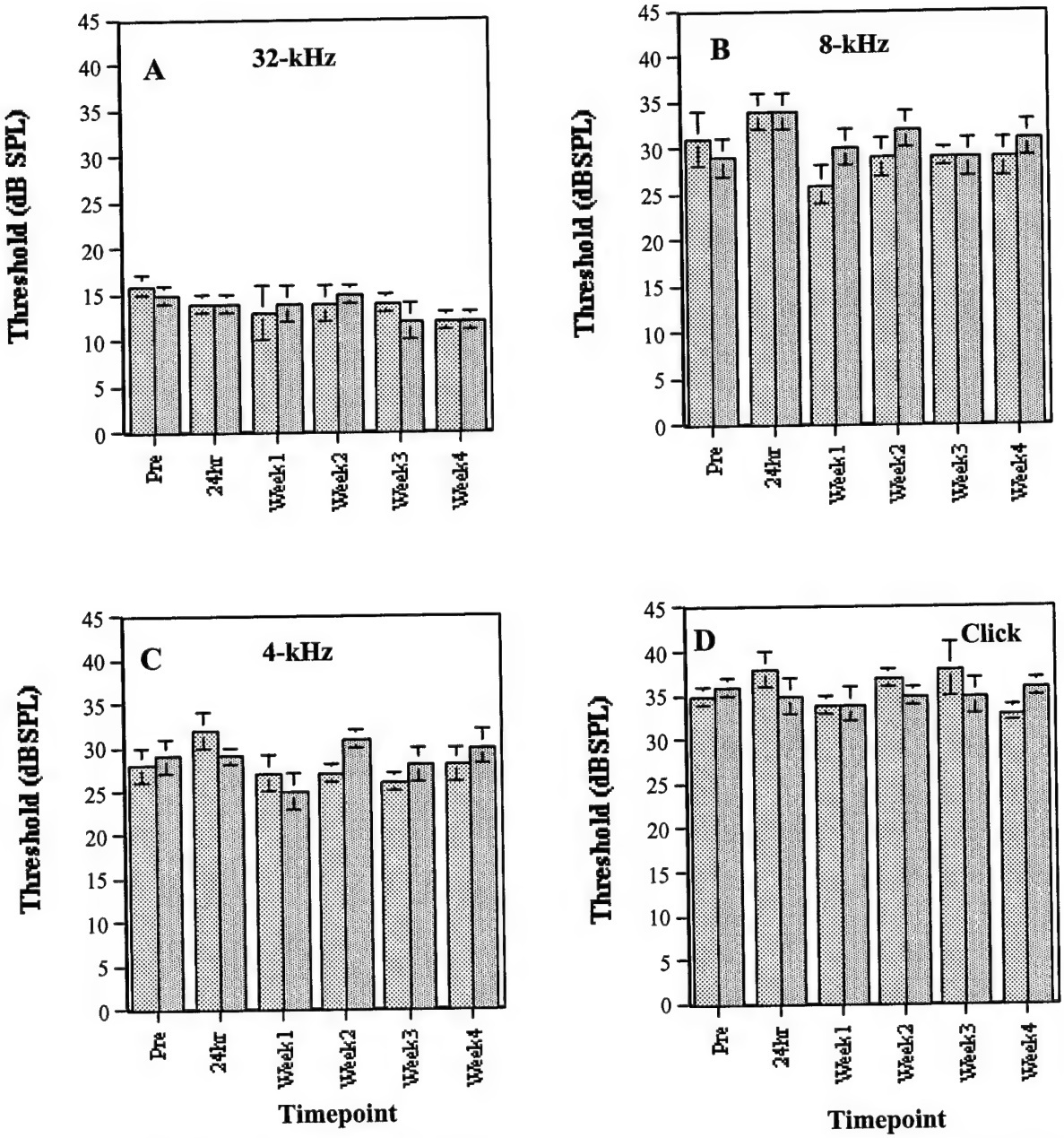


Figure 2-5: PB plus noise stress: Peak II thresholds. Experimental animals administered 5mg/kg PB by gavage weekly for four weeks. Control animals gavaged with equal volume of saline. Immediately after PB or saline administration animals were exposed to 8h of 85dBSPL white noise. Thresholds are expressed as the mean ±SEM from either 5 (controls) or 8 (exp) independent observations (N). A: 32-kHz stimulus; B: 8-kHz stimulus; C: 4-kHz stimulus; D: Click. Timepoints: 24 h before the toxin or saline+ noise (pre); 24 h after the toxin or saline + noise (24h); one to four weeks after exposure to first dose of PB or saline plus noise (Week1-Week4).
Legend: Control Experimental

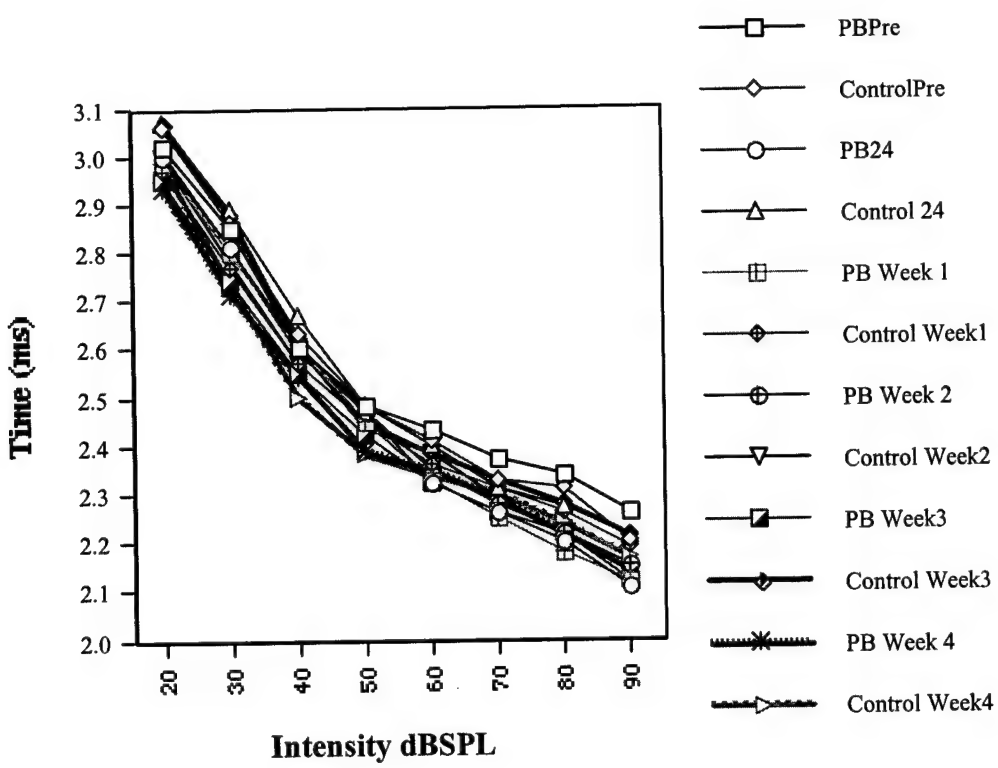


Figure 2-6. 32-kHz Peak II latency/intensity function before and after administration of chronic low-dose PB or saline followed by exposure to 8 hours of noise. Values are expressed as the mean from either 5 (controls) or 8 (test) independent observations (N). All SEMs<0.1 ms (not shown). PB=animals treated with 5 mg/kg PB. Control=animals treated with equal volume of saline. Pre=data obtained 24h prior to administration of first dose of PB or saline and noise exposure; 24=data obtained 24h after first dose of PB or saline and noise; W1-W4=data obtained 6 days after each of the four weekly administrations of PB or saline and exposure to noise.

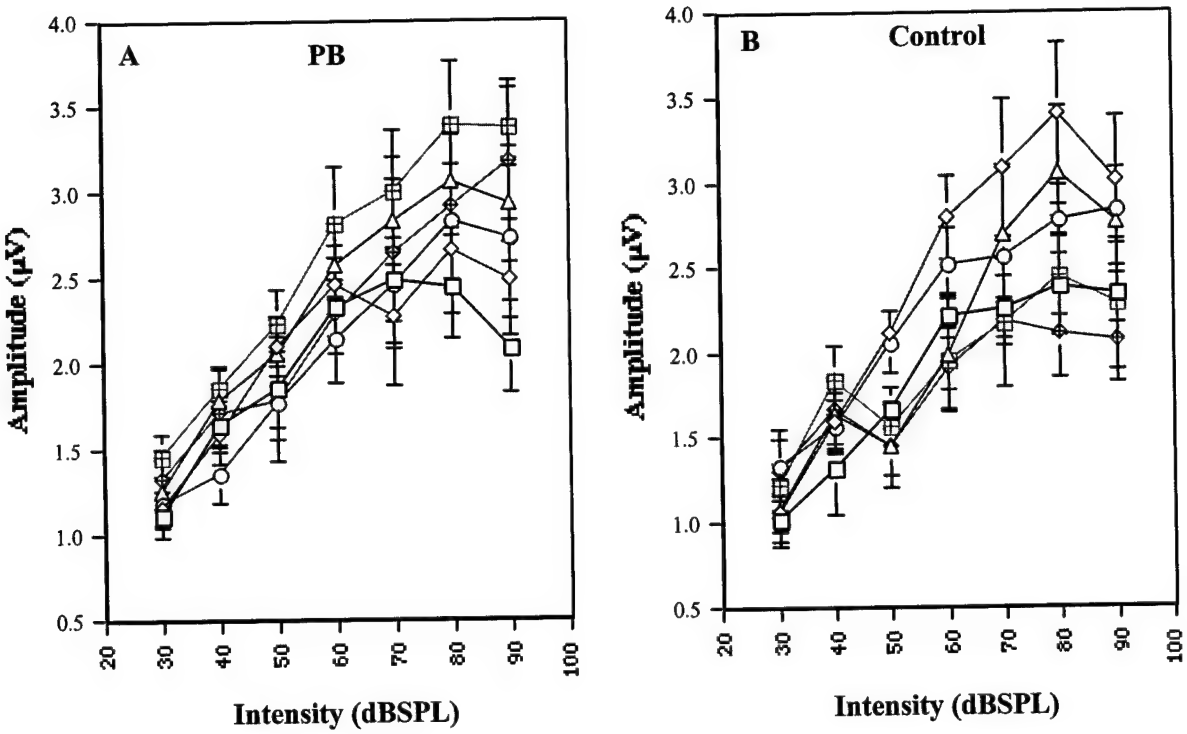


Figure 2-7. 32-kHz Peak II amplitude/intensity function before and after administration of chronic low-dose PB or saline followed by exposure to 8 hours of noise. Values are expressed as the mean ±SEM from either 5 (controls) or 8 (test) independent observations (N). Panel A: animals gavaged weekly with 5mg/kg PB. Panel B: Control animals gavaged with equal volume of saline. Pre=data obtained 24h prior to administraiton of first dose of PB or saline and noise exposure; 24=data obtained 24h after first dose of PB or saline and noise; W1-W4=data obtained 6 days after each of four weekly administrations of PB or saline and exposure to noise. Legend: Pre, 24h, Week 1, Week 2, Week 3, Week 4

DEET + PB + noise stress

Results from the DEET + PB + noise stress study, which are reported here, are based on data obtained from 4 test animals and 3 controls. As noted earlier, because of the time involved in collecting NMR data, we were unable to test all the numbers of animals proposed at each timepoint. One half of the proposed N was tested during a four-week period (Group I), and the second half was tested in a subsequent four-week period (Group II). The data here pertain to Group I. Statistical analysis will not be done until data has been collected from both Group I and II.

Because of a major electrical failure in the evoked potentials unit, the pre and 24-h data was lost. Only data from Week 1 to Week 4 is reported here. The experiment with Group II animals will begin July 9, 2002 and be completed August 9, 2002. At that time, the Group I animal study will be repeated.

Peak II thresholds in the presence of noise

Peak II thresholds for the 32-kHz signal generally varied little across Week 1 to Week 4 (Figure 2-8, Panel A). However, the mean Week-3 threshold for the toxin-treated animals was 4 dB lower than that of the controls. These values are based on Ns of only 4 and 3 animals, respectively; hence, no significance is attached to this difference at this time. For the 8- and 4-kHz signals the Week-1 thresholds were slightly higher in toxin-administered animals than in Week-1 controls, but the thresholds decrease to control values at Weeks 2-4. Compared to the mean values obtained in the DEET and PB studies just discussed, thresholds for the 4- and 8-kHz signals and for the click are, overall, a few dB lower in both the DEET plus PB-treated and control animals. As noted earlier, there was a major equipment failure just after the 24-h data was collected. The unit was returned to the manufacturer where, as part of the repair, the transducers were recalibrated. The lower thresholds for Weeks 1-4 may reflect the recalibration. We will be better able to address this issue after we have tested the second group of animals.

Latency/amplitude

As expected, latencies of the 32-kHz signal decreased with increasing intensity (Figure 2-9). The latency/intensity functions for the 32-kHz signal were somewhat variable across the timepoints, but, again, this variability probably was due to the small N. The actual latency means at each intensity were fairly close to those observed in the PB plus noise stress experiment. It is expected that the statistical analysis will show no differences between the control and test groups or between timepoints.

As was seen in the PB plus noise experiment, the amplitude of the 32-kHz Peak II increased with increasing intensity up to, and including 80 dB (Figure 2-10). As noted above, the decrease in amplitudes that was observed at 90 dB at all timepoints may relate to acoustic properties of the ultrasonic signal.

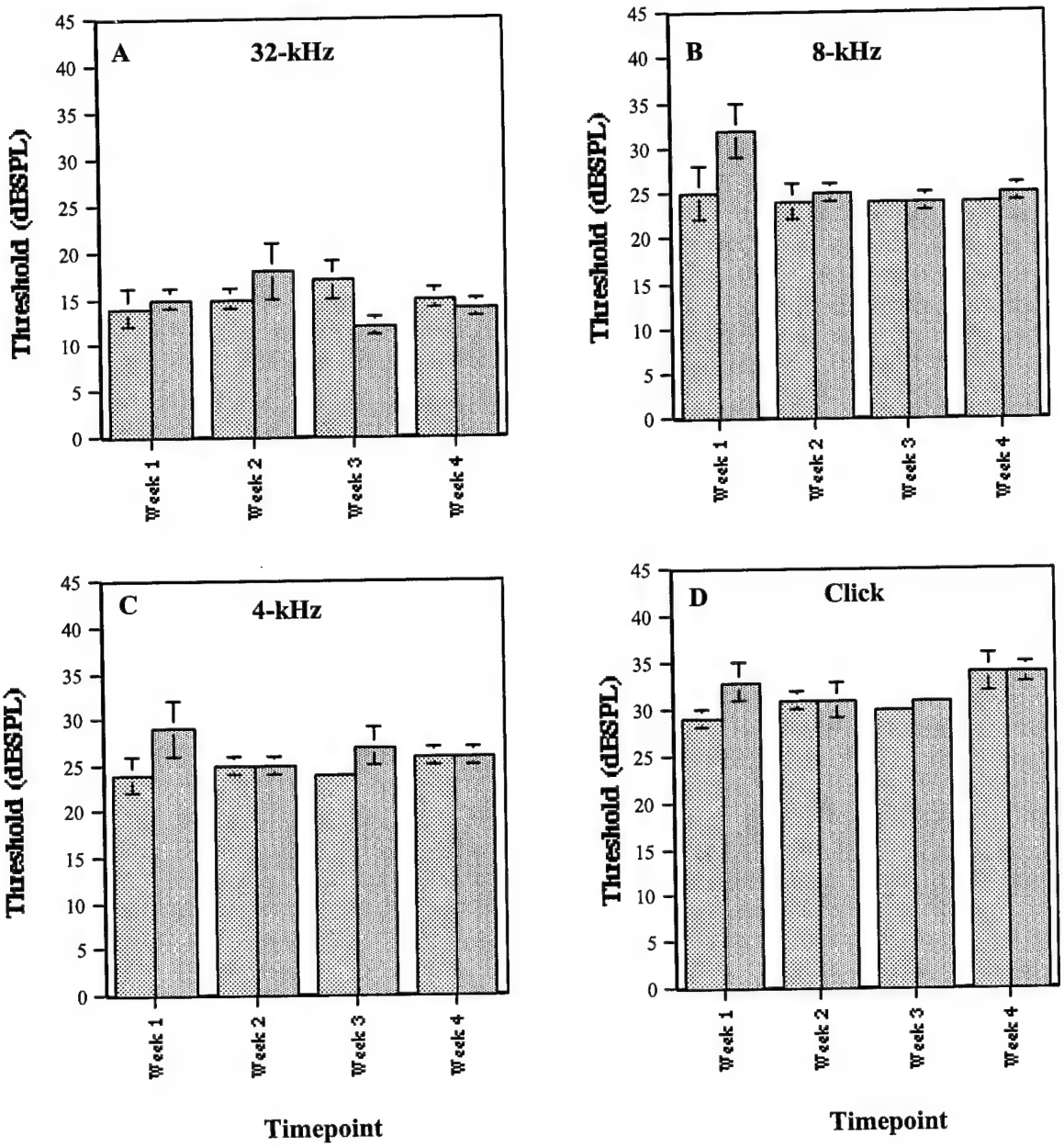


Figure 2-8: PB + DEET + noise stress: Peak II thresholds. Experimental animals administered 225 mg/kg DEET (ip) and 5mg/kg PB by gavage weekly for four weeks. Control animals injected with equa volume of arachis oil and gavaged with equal volume of saline. Immediately after toxin/vehicle administration animals were exposed to 8 h of 85 dBSPL white noise. Thresholds are expressed as the mean ±SEM from either 3 (controls) or 4 (exp) independent observations (N). No error bars: SEM =0. A: 32-kHz stimulus; B: 8-kHz stimulus; C: 4-kHz stimulus; D: Click. Week1-Week4 timepoints: data obtained 6 days after each of the four weekly administrations of toxins or vehicles and exposure to noise. Legend: ▨ CONTROL ▨ EXPERIMENTAL

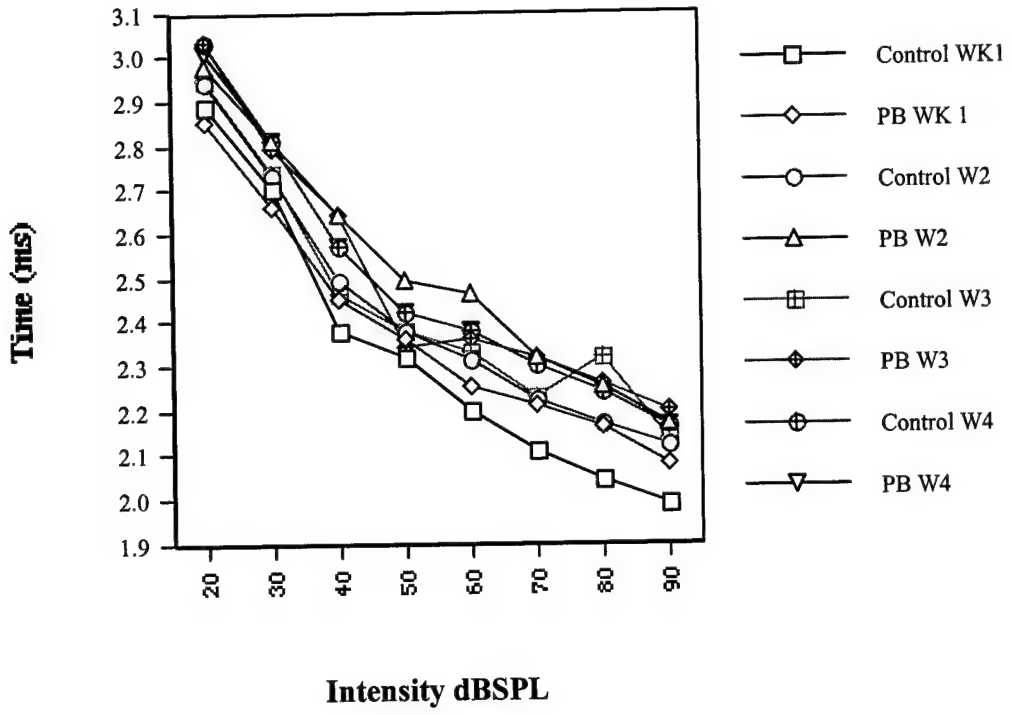


Figure 2-9. 32-kHz Peak II latency/intensity function after administration of chronic low-dose PB + DEET or vehicle followed by exposure to 8 hours of noise. Values are expressed as the mean from either 3 (controls) or 4 (test) independent observations (N). All SEMs<0.1 ms (not shown). PB=animals treated with 225 mg/kg DEET + 5 mg/kg PB. Control=animals treated with equal volumes of vehicles. W1-W4=data obtained 6 days after each of the four weekly administrations of PB or saline and exposure to noise.

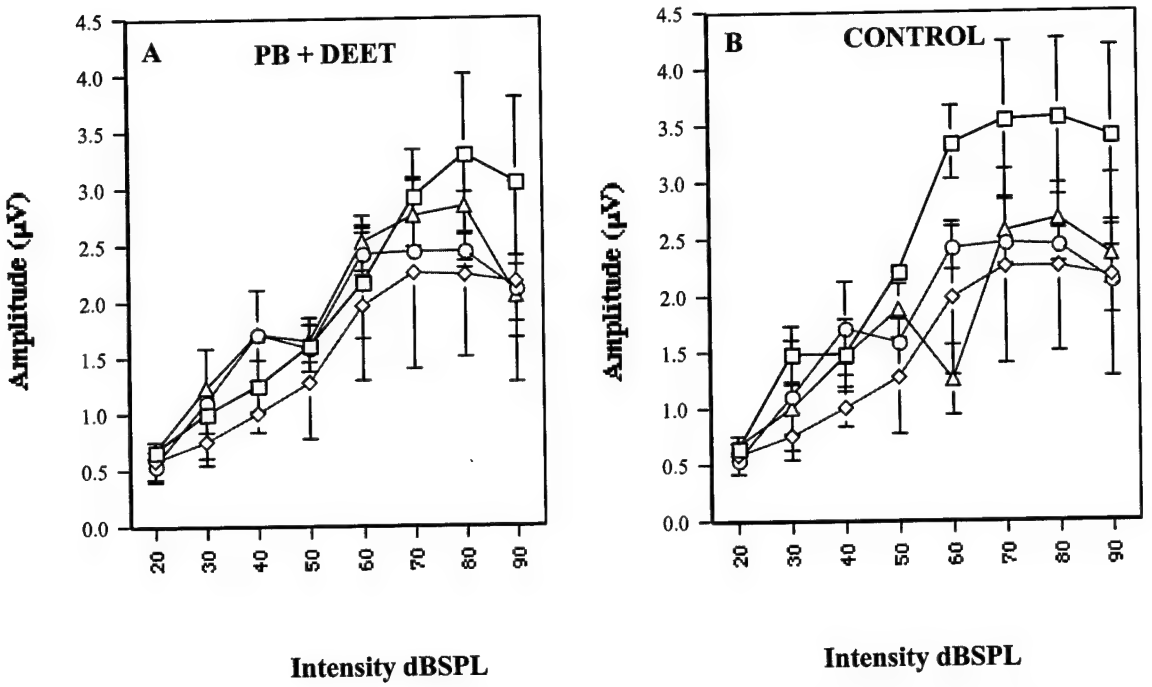


Figure 2-10. 32-kHz Peak II amplitude/intensity function after administration of chronic low-dose PB + DEET or vehicle followed by exposure to 8 hours of noise. Values are expressed as the mean ±SEM from either 3 (controls) or 4 (test) independent observations (N). Panel A: animals injected with 225 mg/kg DEET (ip) and, 10 minutes later, gavaged with 5mg/kg PB. Panel B: Control animals injected with equal volume of arachis oil and, 10 minutes later, gavaged with equal volume of saline. W1-W4=data obtained 6 days after each of four weekly administrations of toxins or vehicles and exposure to noise. Legend: —□— WEEK 1 —◇— WEEK 2 —○— WEEK 3 —△— WEEK 4

Scanning electron microscope (SEM) studies

Previously, we have shown evidence from SEM studies of the cochlear basilar membrane that exposure to 8h of 85 dBSPL broad-band white noise, measured at the level of the rat ear, did not cause hair cell loss or damage in saline-injected control animals. Figure 2-11 is a scanning electron micrograph of inner and outer hair cells located on a mid-cochlear region of the basilar membrane of an animal treated with PB plus noise stress. There is no indication of hair cell loss or damage as a consequence of this treatment. Intact outer and inner hair cells also were observed on the basal and apical regions of the membrane. The integrity of the hair cells was also preserved in animals treated with DEET plus noise stress (not shown). During the past year, we have worked to improve our techniques for preparing and fixing the basilar membrane tissue. We recently purchased a stereomicroscope, which has greatly improved our ability to access both the apex of the cochlea and the round window. We can now gently flush fixative through the entire cochlea during the tissue dissection. We have had some problems with the collapse of the spiral ligament onto the basilar membrane during the fixation/dehydration process. With the better microscope lighting now available to us, portions of the ligament can be pulled away so that hair cells are exposed. This new technique was used with cochleae removed from animals treated with DEET plus PB plus noise. These tissues have been fixed with gluteraldehyde, exposed to OsO4, and dehydrated, but have not been examined yet by SEM.

Corticosterone studies

Three attempts have been made to obtain blood samples for the purpose of measuring corticosterone levels, an indicator of stress, in the animals exposed to noise. In the first attempt, blood was drawn from the saphenous vein of restrained animals, with pre- and post-noise exposure blood being obtained from the same animal. This technique proved to be very stressful to the animals. The corticosterone levels were high under both pre-and post-exposure conditions.

In the second attempt, we used two groups of rats. One group was placed for 30 min in the noise-exposure room, but no noise was presented. At the end of the 30-min period, individual animals were exposed to CO in a closed chamber for a few minutes, removed to another room and decapitated. Blood was quickly drawn into a Microvette tube at the time of decapitation. A separate group of animals was treated in the same way, but they were exposed to 30 minutes of noise during their time in the noise-exposure room. Blood serum was prepared from the blood samples and analyzed for corticosterone levels. Table 2-1 shows the level of corticosterone under conditions of no noise and with 30 min of 85-dBSPL broadband white noise.

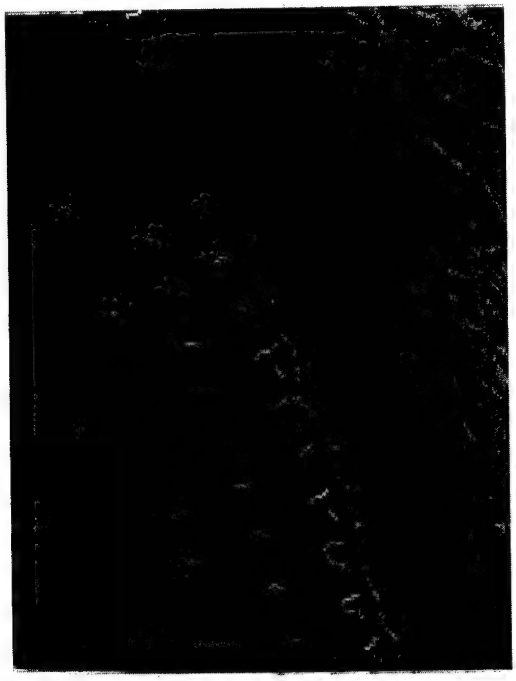


Figure 2-11. SEM of mid-cochlear region of basilar membrane from rat treated with PB and noise stress. V-shaped bundles on left are stereocilia of outer hair cells. At arrow, are stereocilia of inner hair cells.

Table 2-1: Blood serum corticosterone levels after 30 min noise exposure.

Noise condition	Corticosterone (ng/ml) Mean ±SEM
(No noise) Rat 1	107.03
(No noise) Rat 2	202.98
(No noise) Rat 3	199.98
(No noise) Rat 4	38.72
AVERAGE	**137.2±39.7**
(Plus 30 min) Rat 5	212.46
(Plus 30 min) Rat 6	155.68
(Plus 30 min) Rat 7	42.45
(Plus 30 min) Rat 8	263.47
(Plus 30 min) Rat 9	209.40
AVERAGE	**176.7±38.3**

A Student's t test showed no significant difference in these two group means.

A review of the literature had indicated that 30 minutes of noise at the level we were using should induce a rise in corticosterone levels in rats (Ovadia et al., 2001; Amario et al., 1991; Britton et al., 1992; Campeau and Watson, 1997),. A study by Nolan et al., 1977 of diurnal variation of corticosterone in female Wistar rats showed that levels of the stress hormone were low in the first 6 hours of the light cycle and rose to a peak that occurs just prior to the onset of the dark cycle. Our second attempt at measuring corticosterone levels was started at 14.00h, a time, based on the observations of Nolan et al., when the stress hormone would be rising. Normal high levels of stress hormone at this period in the light/dark cycle may have masked any level increases due to noise stress. The high-low spread in our values in Table 2-1 probably reflects variability in stress hormone levels of individual rats during the period when the corticosterone is rising to its peak. We have just repeated this study, using essentially the same technique as was used in the second attempt. However, these measurements were made between 2.5 to 5 hours after the onset of the light cycle (08.30-11.00h); thus, normal corticosterone levels would be expected to be at a low point. These blood serum samples are now being analyzed.

Serum blood levels of DEET and PB

Blood samples were taken from each group of rats at the time of euthanization. Serum was prepared from the samples and stored at -80^0C. New student personnel are now available to analyze these samples.

3. Discussion

The effects of chronic low-dose toxins, both in the absence and presence of noise, were very small, and, in the absence of contrary statistical evidence, we conclude these differences are insignificant. There may be some evidence of slight increases in thresholds at the 24h and Week

1 timepoints in the presence of noise. Because these increases were observed both in the controls and the toxin-treated animals, they probably are temporary threshold shifts that ensue from the noise exposure. The statistical analysis will provide a definitive answer to this issue. The significance of small differences in threshold (<5dB) must be considered in view of the intensity difference limen (IDL) for rats, which is on the order of 2-4 dB. Other factors that may contribute to small differences in threshold include operator technique, individual sensitivity of the test animal, variability and difficulty in interpreting threshold for the 32-kHz signal, which lies in the region of greatest frequency sensitivity for the rat.

Experiments on the effects of PB in the absence of noise and DEET plus PB in the absence of noise were planned but not executed. In order to conserve time, we felt it would be better to do the toxin plus noise studies first. If we did see significant effects with noise, then we would do experiments with the toxins in the absence of noise. We will employ this philosophy for the rest of the study period.

4. Future studies:

The Group II study for DEET plus PB in the presence of noise begins July 9, 2002 and ends August 9, 2002. During the period September 9, 2002 to January 30 2002, the following experiments are planned: Repeat of Group I DEET plus PB in the presence of noise; positive control experiment (toxins under consideration include cis-platin, trimethyltin), establish LD_{50} for sarin; do dose/response study to determine "low dose" for sarin; Group I and II chronic low-dose experiments for sarin plus noise stress.

Presentations and Publications:

Abstracts:

I. R. Bicknell, N. V. Reo, L. Prochaska, M. Forquer, L. Shroyer, A. Neuforth, and T. Young. "ABR, NMR, and energy metabolism studies of the effects of long-term exposure to low doses of DEET in the rat". Annual meeting of the Association for Research in Otolaryngology (ARO), St. Petersburg Beach, FL, February 2002.

PROJECT 2, SECTION B: NMR Studies (PI: Nicholas V. Reo)

1. Chronic low-dose study: DEET +/- noise stress (225 mg/Kg DEET by ip injection once weekly for 4 consecutive weeks; noise administered for 8 h once weekly).

Previous quarterly reports have addressed this study in detail. This was the first series of experiments we conducted using the chronic low-dose protocol, and we were still in the process of debugging problems. Thus we found it necessary to repeat some experiments to increase the n-values; these additional data were collected during March and April 2002. The following is a summary of the results.

NMR measurements in vivo were performed at three timepoints during the experimental protocol: pretreatment, and at 2 and 4 weeks post-treatment. The animals were divided into four groups: Control (-)stress (n=8), DEET (-)stress (n=8), Control (+)stress (n=10), and DEET (+)stress (n=12). Relative signal intensities were measured in rat brain for the following metabolites:

^{1}H spectra: choline (Cho), creatine (Cr), and N-acetylaspartate (NAA).
^{31}P spectra: adenosine triphosphate (ATP) and phosphocreatine (PCr).

The ^{1}H peaks were normalized to the Cr signal intensity and expressed as a ratio relative to Cr. Cr is uniformly distributed in brain and is usually unaffected by pathological processes. The ^{31}P peaks were normalized to total phosphorus by integrating the entire ^{31}P brain spectrum. The ratio of NAA:Cr is thought to represent functional neuronal mass, while the ratio of PCr:ATP is a measure of the cellular energy status. Cho reflects amounts of phosphocholine and glycerophosphocholine that are associated with phospholipid metabolism.

The metabolite ratios do not necessarily represent the tissue concentration ratios since the NMR data were not acquired under 'quantitative conditions' (i.e., full T_1 relaxation). These data are, however, useful for comparative purposes to delineate differences between groups.

TABLE 2-2

Group	Cho/Cr	NAA/Cr	PCr/ATP
Control (-)stress	1.86 ± 0.15 (n=4)	1.66 ± 0.14 (n=4)	1.47 ± 0.09 (n=6)
DEET (-)stress	1.56 ± 0.08 (n= 5)	2.40 ± 0.34 (n=5)	1.57 ± 0.04 (n=8)
Control (+)stress	1.50 ± 0.10 (n=8)	1.77 ± 0.17 (n=8)	1.59 ± 0.08 (n=9)
DEET (+)stress	1.52 ± 0.16 (n=10)	1.72 ± 0.24 (n=10)	1.71 ± 0.09 (n=10)
All groups combined	1.57 ± 0.07 (n=27)	1.85 ± 0.13 (n=27)	1.60 ± 0.04 (n=33)

The pretreatment measurements were conducted prior to DEET or noise stress exposure. Thus animals in all groups are not distinct at this measurement timepoint. A two-way ANOVA was conducted with the pretreatment data as the dependent variable to assess the variance in the four groups. The two independent factors were the group identifiers, treatment and stress. This statistical analysis showed no significant differences among the groups prior to treatment or

stress ($p < 0.05$). Table 2-2 shows the metabolite ratios (mean ± SE) measured in rat brains in vivo at the pretreatment timepoint for each experimental group. The Cho/Cr and NAA/Cr ratios were measured from the ^{1}H NMR spectra while the PCr/ATP ratios were measured by ^{31}P NMR. In some spectra the signal-to-noise ratio (S/N) was poor and prohibited an accurate measure of signal intensities. These data were eliminated from the analyses. This accounts for the differences in n-values for some of the experimental groups.

A repeated measures ANOVA was conducted to test whether treatment and/or stress produced any significant changes in the data over times post-treatment. Here the data obtained at pretreatment, 2 wk, and 4 wk were repeated measurements (dependent variables) with treatment and stress as independent factors. All measurements (Cho/Cr, NAA/Cr, and PCr/ATP) showed no effects of either DEET exposure alone or the combination of DEET + stress ($p < 0.05$). Noise stress alone, however, produced a significant effect on the Cho/Cr data as determined by this repeated measures ANOVA ($p=0.05$). The Cho/Cr ratios averaged over all timepoints (mean ± SE) for animals exposed to stress (1.50 ± 0.06, n=51) were slightly lower than the value for animals not exposed to stress (2.02 ± 0.16, n=24). If this does reflect some changes in membrane components, then the effect was not large enough to impact energy metabolism since PCr:ATP ratios were not affected.

These NMR data corroborate the ABR measurements and the measurements of mitochondrial function: DEET exposure in rats, with or without noise stress, does not compromise brain function or cellular energy metabolism.

2. Chronic low-dose study: pyridostigmine bromide + noise stress

Rats were separated into two groups, PB-treated (n=8) and control (n=6). Treated rats were administered pryridostigmine bromide (PB) at 5 mg/Kg (in normal saline) by intragastric gavage. Controls were given an equal volume of vehicle solution. The treatment and stress protocol was identical to that described previously for the "chronic low-dose study" (see Section A). Briefly, rats were treated with PB or vehicle and exposed to 8 h of noise stress once weekly for four consecutive weeks. During this timecourse ABR and NMR experiments were conducted in vivo. At the end of the 4-week protocol, the rats were sacrificed for in vitro analyses of brainstems (NMR of tissue extracts and mitochondrial studies).

NMR In Vivo. NMR studies in vivo were conducted in both rat head (brain) and hind leg (muscle). Proton (^{1}H) and ^{31}P NMR measurements were made in the head, while ^{31}P and ^{13}C NMR spectra were acquired from the leg muscle. NMR measurements were performed at three timepoints during the experimental protocol: pretreatment, and at 2 and 4 weeks post-treatment. The ^{1}H and ^{31}P spectral analyses were identical to that described above for the DEET study. The ^{13}C NMR spectra of muscle primarily show signals due to lipids. These spectra will be compared between experimental groups, but these analyses are not yet completed.

The pretreatment measurements were conducted prior to PB or noise stress exposure. Thus animals in all groups are not distinct at this measurement timepoint. A one-way ANOVA was conducted with the pretreatment data as the dependent variable to assess the variance in the two groups. The independent factor was the group identifier, PB-treated or control. This statistical analysis showed no significant differences among the groups prior to treatment or stress ($p < 0.05$). Tables 2-3 and 2-4 show the metabolite ratios (mean ± SE) measured in rat brain and muscle in vivo, respectively, at the pretreatment timepoint for PB-treated and control groups.

The Cho/Cr and NAA/Cr ratios were measured from the ^{1}H NMR spectra while the PCr/ATP ratios were measured by ^{31}P NMR. In some spectra the signal-to-noise ratio (S/N) was poor and prohibited an accurate measure of signal intensities. These data were eliminated from the analyses. This accounts for the differences in n-values for some of the experimental groups.

TABLE 2-3. NMR Metabolite Measurements of Rat Brain

Group	Cho/Cr	NAA/Cr	PCr/ATP
Control (+)stress	1.38 ± 0.10 (n=4)	1.12 ± 0.08 (n=4)	1.62 ± 0.07 (n=5)
PB (+)stress	1.41 ± 0.14 (n=6)	1.34 ± 0.07 (n=6)	1.64 ± 0.08 (n=8)
All groups combined	1.39 ± 0.09 (n=10)	1.25 ± 0.06 (n=10)	1.63 ± 0.05 (n=13)

TABLE 2-4. NMR Metabolite Measurements of Rat Muscle

Group	PCr/ATP
Control (+)stress	2.33 ± 0.09 (n=6)
PB (+)stress	2.42 ± 0.07 (n=8)
All groups combined	2.38 ± 0.06 (n=14)

The pretreatment Cho/Cr and NAA/Cr ratios in rat head (Table 2-3) seem slightly lower than the corresponding values obtained from the DEET study (Table 2-2). The animals for the PB study were slightly younger than those used for the DEET study, and different diets were used for the two studies. Whether these factors could be the cause for these metabolite differences is unclear. The tissue energy status, however, is not different among all these animals as evidenced by the similar values obtained for the rat head PCr/ATP data from the two studies (approximately 1.6). Of course, the PCr/ATP values for brain and skeletal muscle are different whereby the muscle values are higher as expected (Table 2-3 vs Table 2-4).

The ^{31}P NMR spectra were not obtained under conditions of full T_1 relaxation and thus the data do not reflect accurate molar ratios. This is not a problem, however, since we are concerned only with relative changes in the values due to treatment. The molar ratios of PCr:ATP in adult rat brain as measured by ^{31}P NMR have been reported to be in the range 1.85 - 2.10 (Liu, et al., 1991; Shoubridge, et al., 1982; Tofts and Wray, 1985). Since the T_1 relaxation time of PCr is > the T_1 of ATP, then as expected our ratio of signal intensities is < the molar ratio values reported for PCr:ATP.

A repeated measures ANOVA was conducted to test whether PB treatment produced any significant changes in the data over times post-treatment. Here the data obtained at pretreatment, 2 wk, and 4 wk were repeated measurements (dependent variables) with treatment as an independent factor. These mean ± SE values for each metabolite ratio are displayed in Figure 2-12. All measurements (Cho/Cr, NAA/Cr, and PCr/ATP) showed no effects due to PB exposure for both brain and muscle ($p < 0.05$). These data corroborate the ABR studies and measurements

of mitochondrial function. Together these studies show that exposure of rats to PB and noise stress over a 4-week period does not significantly affect brain function or energy metabolism.

NMR In Vitro. Brainstems were obtained by microwave fixation (see #4 below) and stored in liquid N_2 for subsequent analyses by high-resolution NMR. We are currently in the process of preparing these tissues for analyses (PB-treated, n=6; control, n=3). Data are not available for discussion at this time.

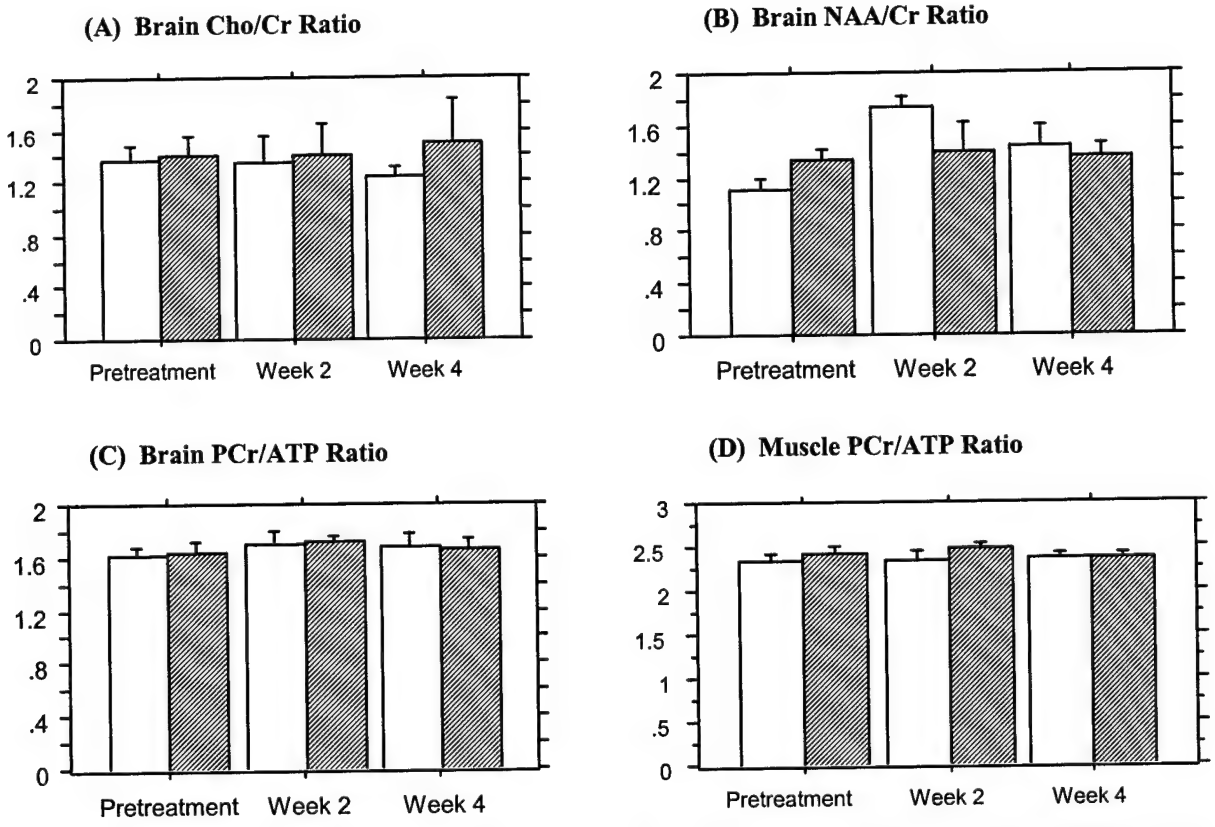


Figure 2-12. NMR measured metabolite ratios (Mean ± SE) in rat brain and leg muscle in vivo for PB-treated (hatched bars) and control (open bars) groups. Measurements are before (pretreatment) and at 2 and 4 weeks during the chronic exposure protocol involving PB or saline plus noise stress. (A), (B), and (C) are the Cho/Cr, NAA/Cr, and PCr/ATP ratios, respectively, measured in rat brain, while (D) shows the muscle PCr/ATP ratios measured in the same rats.

3. Chronic low-dose study of drug synergism: DEET + pyridostigmine bromide + noise stress.

This study began on May 21, 2002 and it is currently in progress. Briefly, this study involves exposure of rats to a combination of DEET (225 mg/Kg, ip) + PB (5 mg/Kg, ig) + 8 h of noise stress administered once weekly for 4 consecutive weeks. The ABR, NMR, and mitochondrial studies are conducted using the identical protocol as described previously for other chronic low-dose studies. Data from these studies will be provided in a future quarterly report.

4. Optimized experimental procedures for NMR analyses of brainstem extracts in vitro.

At the completion of the 4-week protocol involving ABR and NMR studies in vivo, animals are sacrificed for in vitro analyses of brainstems. These include preparation of chemical extracts for high-resolution NMR analyses and preparation of brainstem mitochondria for studies of cellular energetics (Project 2, Section C).

For the NMR studies, rat brains are prepared by microwave fixation using a Metabolic VivoStat (Model S6G, Cober Instruments). This instrument provides 6 KW of microwave energy to rapidly fix the rat brain in <2 sec. This preserves labile energy metabolites that can then be chemically extracted from the tissue for quantitative analyses. In our initial studies involving DEET ± stress we discovered that the microwave method was ineffective because the rats were too large (>400 g). Thus in subsequent studies we used slightly younger animals and maintained the rats on a diet specially formulated for toxicology studies. This has resulted in smaller animals and better results for the microwave tissue fixation.

We have conducted studies to test the precision of our chemical extraction techniques and our NMR analyses. After finding that a perchloric acid extraction method yielded variable results, we instead chose to use an extraction procedure involving chloroform/methanol/water as a solvent system. The aqueous phase is then separated and lyophilized to dryness. The dried extract is reconstituted in D_2O and analyzed by high-resolution ^{1}H and ^{31}P NMR spectroscopy.

To test the precision of the extraction procedures we combined brain tissues obtained by microwave fixation from six rats. The pooled samples were frozen in liquid N_2, ground to a powder, homogenized, and then divided into four equal portions. Each portion was subjected to chemical extraction and ^{31}P NMR analysis. The results yielded a PCr/ATP ratio of 1.80 ± 0.2 (mean ± SD, n=4). The spectra were also calibrated to obtain quantitative data yielding concentrations for PCr and ATP as 2.10 ± 0.18 and 1.16 ± 0.12 umol/g tissue, respectively (mean ± SD, n=3). The standard deviations in all these data (about 10%) are within our experimental limits. Further experiments are in progress to specifically analyze the brainstem instead of whole brain samples. We have determined that the minimum amount of sample required to obtain quantitative data is about 100 mg. Thus we will be able to analyze brainstems from individual animals since samples typically range from 100-300 mg depending upon the effectiveness of the microwave fixation method.

5. Constructed a new NMR probe for studies of rat leg muscle in vivo.

The NMR studies in vivo are conducted on rat head and leg using surface coils. The head experiments provide spectra of brain while the leg experiments provide spectra of skeletal muscle. We had originally constructed two surface coils contained in a single probe. One surface coil was positioned on the rat's head and the other was positioned on the leg muscle. Thus both brain and muscle experiments can be conducted in a given animal sequentially without removing the probe or animal from the NMR magnet. This procedure, however, proved to be difficult in practice since the animals were rather large (≥400 g). The leg surface coil was too tight against the animal and sometimes produced pressure sores on the leg. These large animals also caused problems with the microwave fixation of brain tissues. Such problems prompted us to make some changes in the experimental protocol to reduce the size of the rats used in

experiments (i.e., use younger animals and adjust the diet). These changes were detailed in a previous quarterly report and they have resulted in smaller animals.

With regard to the NMR studies in vivo, we also decided to construct separate probes for the head and muscle experiments. This was done to optimize the procedures for each experiment and to minimize the problems we were having with the leg experiments. The experimental procedure involves placing the rat in the 'head coil probe' to acquire ^{1}H and ^{31}P NMR spectra from the head. The probe is then removed from the magnet and the rat is positioned into the leg coil probe for muscle NMR data acquisition. The leg probe now has extra space to better accommodate the rat. This has alleviated the problems with leg sores.

Section C: Energy Metabolism, Lawrence J. Prochaska, PhD., P. I.

1. *The Effects of DEET and DEET with Noise Stress on Mitochondrial Respiratory Control and Electron Transfer Activities.*

Rats were treated with 37.5% of the LD_{50} dose of DEET or alternatively with 37.5% of the LD_{50} dose of DEET in the presence of noise stress as described in the protocol presented in the Auditory Biology portion of this report (Section 2A). Thirty days after the initial treatment with DEET or DEET plus noise stress, animals were sacrificed and mitochondria were isolated from brain stems using density gradient centrifugation. Energy coupling ratios using two different substrates (Table 2-5) and partial reactions of respiratory chain electron transfer in the absence of pH and membrane potential gradients were also measured (Table 2-6).

Table 2-5 shows that there was little or no effect of long-term exposure of rats to DEET or to DEET with noise stress on the respiratory control ratios of isolated mitochondria when using succinate (FAD-linked) or pyruvate and malate (NADH-linked) as substrates. This result suggests that DEET has no effect on the ability of mitochondria to make ATP and that the inner membrane permeability of mitochondria was unaffected by DEET treatment. The total yield of isolated mitochondria from brain stem tissue was similar in all animals, emphasizing that there was no decrease in mitochondrial integrity in any of the tissues studied. However, there appears to be a loss of coupled (ADP and Pi) electron transfer activity from NADH to oxygen in DEET-treated animals (see discussion below).

Table 2-5. The Effects of a 37.5% LD_{50} Dose of DEET on Rat Brain Stem Mitochondrial Energy Coupling

Treatment	Substrate	Activity (nmoles O_2) (mg protein-min)[a]	Respiratory Control Ratio
Control			
	Succinate	**22 ± 1**	
	+ADP	61 ± 7	2.8 ± 0.3
	Pyruvate/malate	**12 ± 6**	
	+ADP	37 ± 6	3.5 ± 1.0
Control with Noise Stress			
	Succinate	13 ± 2	
	+ADP	47 ± 4	3.7 ± 1.0
	Pyruvate/malate	**10 ± 3**	
	+ADP	38 ± 6	4.0 ± 0.8
DEET-Treated (30 days after injection)			
	Succinate	18 ± 4	
	+ADP	68 ± 14	3.8 ± 1.1
	Pyruvate/malate	**11 ± 5**	
	+ADP	29 ± 6	3.6 ± 0.7
DEET-Treated (30 days after injection) with Noise Stress			
	Succinate	14 ± 5	
	+ADP	55 ± 6	4.2 ± 1.8
	Pyruvate/malate	**11 ± 8**	
	+ADP	32 ± 8	3.6 ± 1.7

[a]All assays were performed in a buffer consisting of 10 mM KH_2PO_4, 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 1mM EGTA, pH 7.4. Substrate concentrations used were 3 mM succinate, 3 mM pyruvate and 1.5 mM malate, and 0.2 mM ADP. Error measurements are standard deviations. The number of preparations was 10 for control, 4 for control with noise stress, and 2 for both DEET-treated with and without noise stress. Each assay was performed at least six times for each preparation.

Table 2-6 shows the effects of long-term exposure of DEET and DEET with noise stress on partial electron transfer reactions of the mitochondria respiratory chain when assayed in the absence of a membrane potential or a pH gradient. Neither cytochrome oxidase activity nor succinate dehydrogenase activity of brain stem mitochondria was affected by treatment of rats with DEET or DEET with stress. Again, the yield of mitochondria from the brain stem tissue was the same in all animals. There was a 15% decrease in electron transfer activity from NADH (from NADH dehydrogenase through the cytochrome bc_1 complex) to ferricyanide in both DEET-treated and DEET-treated with stress animals, suggesting a possible site of inhibition in Complex I of the respiratory chain. The specificity of inhibition of electron transfer activity in NADH dehydrogenase was assessed further by measuring electron transfer from NADH to ubiquinone (Complex I). Table II shows that there is no inhibition in the rate of electron transfer in Complex I (NADH $\Rightarrow$ UQ) induced by DEET or DEET plus stress. The observed inhibition in NADH $\Rightarrow$ ferricyanide is currently unresolved. In addition, there was a significant decrease in NADH $\Rightarrow$ ferricyanide activity in stressed control animals, suggesting that the stressor (sound) alone may be perturbing some mitochondrial activities. This effect is currently under investigation.

Table 2-6. The Effects of a 37.5% LD_{50} Dose of DEET on Rat Brain Stem Mitochondrial Electron Transfer Activity

Treatment	Specific Activities (nmol/min/mg-protein)[a]			
	Cyt. C ⇒ O_2	**Suc ⇒DCIP**	**NADH ⇒ FeCN**	**NADH ⇒ UQ**
I. Control				
	248 ± 20	23 ± 1	1973 ± 80	66 ± 3
II. Control with Noise Stress				
	250 ± 31	28 ± 1	1234 ± 62	59 ± 7
III. DEET-Treated (30 days after injection)				
	253 ± 18	24 ± 1	1660 ± 50	68 ± 6
IV. DEET-Treated (30 days after injection) with Noise Stress				
	231 ± 12	25 ± 1	1642 ± 52	67 ± 6

[a]Activity measurements are presented in nmoles oxygen consumed, nmoles DCIP reduced, nmoles FeCN reduced or nmoles NADH oxidized/mg mitochondrial protein –min. Error measurements are standard deviations. The number of preparations was 10 for control, 4 for control with noise stress, and 2 for both DEET-treated with and without noise stress (6 assays for each preparation).

2. *The Effects of DEET and DEET with Noise Stress on Isolated Mitochondrial Cytochrome c Content.*

Previous work by others has shown that translocation of cytochrome *c* from mitochondria into the cytoplasm is a marker for cells arriving at a checkpoint in the apoptotic pathway of cell death (Green and Reed, 1998). Figure 2-13 shows the cytochrome *c* content of our isolated mitochondria as detected by immunoblotting using our polyclonal antibodies to cytochrome *c*. There are no statistically significant differences in the cytochrome *c* content of mitochondria isolated from control animals, control animals treated with noise stress, DEET-treated animals, and DEET-treated animals which also received noise stress. This result taken together with the data where similar yields of isolated mitochondria were obtained from brain stems in control and treated animals suggests that DEET and DEET with noise stress does not induce significant apoptosis in rat brain stem cells during the time period studied.

Figure 2-13. The Effects on DEET and DEET with Noise Stress on the Cytochrome *c* Content of Isolated Rat Brain Stem Mitochondria.

Electrophoresis, protein transfer to PVDF, and blot blocking were performed as described in Ogunjimi et *al.* (2000) and the blot was developed using polyclonal antibodies against cytochrome *c* (1:1000 dilution) (Kirken et *al.*, 1995). Color development was facilitated using alkaline phosphatase linked to goat antirabbit IgG. Lane 1 contains 12 ngm pure cytochrome c; Lane 2, 2.5 μgm control mitochondria; Lane 3, 2.5 μgm mitochondria from DEET-treated animals; Lane 4, 2.5 μgm mitochondria from stressed control animals; Lane 5, 2.5 μgm mitochondria from animals treated with DEET and noise stress. Scanning densitometry showed no significant differences between lanes 2-5.

Summary:

DEET was used extensively as an insect repellent by personnel engaged in the Gulf War and is thought to be integral in the development of the Gulf War Syndrome, a loosely defined family of neurological and neuromuscular symptoms. Patients with the syndrome exhibit both acute and latent symptoms that include fatigue, weakness, and muscle wasting. The observed latent symptoms could be due to long-term decreases in mitochondrial functioning within brain stem cells. One of the goals of this study was to investigate whether relevant dosages of DEET and DEET in the presence of stress affected energy metabolism in the brain stem. Towards this goal, mitochondria were isolated from brain stems of treated animals and mitochondrial energy coupling and electron transfer activities measured.

The results of our studies show that the 37.5% LD_{50} dose of DEET had little or no effect on brain stem mitochondrial energy coupling and electron transfer activities. The effect of DEET and DEET with stress on energy coupling was assessed using FAD-linked substrates, succinate, and NADH linked substrates, pyruvate and malate. The two different substrates were used to examine energy coupling through different sites of ATP synthesis in the mitochondrial respiratory chain to determine if DEET had any effect on mitochondrial membrane permeability. Our results showed that DEET had no effect on energy coupling ratios (as monitored by the respiratory control ratio), but had a small effect on the coupled (ADP + Pi) rate of electron transfer using NADH-linked substrates. This result may be significant due to Complex I of the respiratory chain being a major site of oxidative damage in the cell.

The effects of DEET and DEET with noise stress treatments on partial reactions of the respiratory chain were also investigated to assess if the treatments had any site-specific inhibition of mitochondrial electron transfer. Assays were designed to assess specific portions of the respiratory chain activity, but contain redundant electron transfer pathways to provide an internal check for experiments. Significant decreases in electron transfer rates in the absence of any membrane potential or pH gradient were observed only in the NADH to FeCN assays in both stressed controls and DEET treated animals, again, suggesting that DEET and/or stress induced a change in Complex I electron transfer activity. When the mitochondria were assayed with a more specific Complex I assay (NADH to ubiquinone), no inhibition was observed in all animals including those treated with DEET, suggesting that Complex I was not the site of inhibition in the NADH to FeCN assay.

The endogenous cytochrome *c* content of our mitochondrial preparations was measured to determine if the mitochondria were exhibiting the initial signs of undergoing apoptosis. Cytochrome *c* has been shown to be translocated into the cytoplasm during the onset of apoptosis. Our results showed that none of the treatments affected the cytochrome c content of isolated mitochondria. The absolute yield of mitochondria from brain stem tissue of all animals was similar, suggesting no major mitochondrial damage was induced by either stress or DEET. Thus, these results taken together suggest that neither DEET nor stress induced major changes in brain stem tissue at the cellular or sub cellular level.

One conclusion of our results is that DEET in the presence or absence of stress does little to alter brain stem energy metabolism. DEET and/or stress had no major effects on isolated brain stem mitochondrial functioning. Another conclusion is that it is unlikely that DEET or stress can induce apoptosis of brain stem cells. The cytochrome c content of isolated brain stem mitochondria was unaffected by DEET and/or stress treatments. Therefore, it is unlikely that DEET alone can account for the symptoms described in the Gulf War Syndrome.

3. *The Effects of Pyridostigmine Bromide on Mitochondrial Energy Coupling (Respiratory Control) and Electron Transfer Activities.*

Rats were treated with 5 mg pyridostigmine bromide (PB)/ Kg body weight in the presence of noise stress as described in the auditory portion of this project. Thirty days after the initial treatment, animals were sacrificed and mitochondria were isolated from brain stems using density gradient centrifugation. Energy coupling ratios using two different substrates (Table 2-7)

and partial reactions of respiratory chain electron transfer in the absence of pH and membrane potential gradients (Table 2-8) were measured.

Table 2-7 shows that there was little or no effect of long-term exposure of PB with noise stress on the respiratory control ratios of isolated mitochondria when using succinate (FAD-linked) or pyruvate and malate (NADH-linked) as substrates. This result suggests that PB has no effect on the ability of mitochondria to make ATP and that the inner membrane permeability of mitochondria was unaffected by PB treatment. The total yield of mitochondria from the brain stem tissue was similar in PB-treated with stress and control animals treated with stress, emphasizing that there was no decrease in integrity of the mitochondria in the tissue upon PB with stress treatment.

Table 2-7 The Effects of a 5mg/Kg Body Weight Dose of PB on Rat Brain Stem Mitochondrial Energy Coupling

Treatment	Substrate	Activity (nmoles O_2) (mg protein-min)[a]	Respiratory Control Ratio
I. Control			
	Succinate	**22 ± 1**	
	+ADP	61 ± 7	2.8 ± 0.3
	Pyruvate/malate	**12 ± 6**	
	+ADP	37 ± 6	3.5 ± 1.0
Control + Noise Stress			
	Succinate	13 ± 2	
	+ADP	47 ± 4	3.7 ± 1.0
	Pyruvate/malate	**10 ± 3**	
	+ADP	38 ± 6	4.0 ± 0.8
PB-Treated + Noise Stress (30 days after injection)			
	Succinate	17 ± 3	
	+ADP	61 ± 12	3.8 ± 0.9
	Pyruvate/malate	**8 ± 4**	
	+ADP	32 ± 7	5.2 ± 2.9

[a]All assays were performed in a buffer consisting of 10 mM KH_2PO_4, 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 1mM EGTA, pH 7.4. Error measurements are standard deviations. The number of preparations was 10 for control, 4 for control with noise stress, and 2 for PB-treated with noise stress. Each assay was performed at least six times for each preparation.

The effects of long-term exposure of PB with noise stress on partial electron transfer reactions of the mitochondrial respiratory chain were also determined (Table 2-8). Neither cytochrome oxidase activity nor succinate dehydrogenase activity of brain stem mitochondria was affected by treatment of rats with PB with stress. Again, the yield of mitochondria from brain stem tissue was the same in all treated animals. Significant decreases in electron transfer were observed in the NADH to FeCN assay for both stressed controls and animals treated with stress and PB. However, the site-specific assay for Complex I (NADH to UQ) showed no differences between the treated and control animals.

Table 2-8. The Effects of a 5 mg/Kg Body Weight Dose of PB on Rat Brain Stem Mitochondrial Electron Transfer Activity

Treatment	Specific Activities (nmol/min/mg-protein)[a]			
	Cyt. C ⇒ O_2	**Suc ⇒DCIP**	**NADH ⇒ FeCN**	**NADH ⇒ UQ**
I. Control				
	248 ± 20	23 ± 1	1973 ± 80	66 ± 3
II. Control with Noise Stress				
	250 ± 31	28 ± 1	1234 ± 62	59 ± 7
III. PB-Treated with Noise Stress (30 days after injection)				
	235 ± 7	35 ± 3	1527 ± 60	62 ± 7

[a]Activity measurements are presented in nmoles oxygen consumed, nmoles DCIP reduced, nmoles FeCN reduced or nmoles NADH oxidized/mg mitochondrial protein –min. Error measurements are standard deviations. The number of preparations was 10 for control, 4 for control with noise stress, and 2 for PB-treated with noise stress. Each assay was performed at least six times for each preparation.

4. ***The Effect of PB with Noise Stress on Isolated Mitochondrial Cytochrome c Content.***

Figure 2-14 shows the cytochrome *c* content of our isolated mitochondria as detected by immunoblotting using our polyclonal antibodies to cytochrome *c*. There are no statistically significant differences in the cytochrome *c* content of mitochondria isolated from control animals, control animals treated with noise stress, DEET-treated animals which received noise stress, and PB-treated animals which also received noise stress. This result taken together with the data where similar yields of isolated mitochondria were obtained from brain stems in control and treated animals suggests that PB with noise stress does not induce significant apoptosis in rat brain stems during the time period studied.

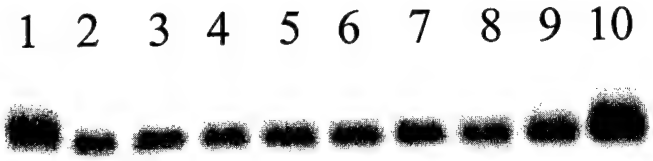


Figure 2-14. The Effects on PB with Noise Stress on the Cytochrome *c* Content of Isolated Rat Brain Stem Mitochondria.

All conditions were as described in Figure 2-13. Lanes 1 and 10 contain 12 ngm pure cytochrome c; Lanes 2 and 3, 2.5 μgm mitochondria from animals treated with PB and stress; Lanes 4 and 5, 2.5 μgm mitochondria from animals treated with PB and stress; Lanes 6 and 7, 2.5 μgm mitochondria from animals treated with DEET and stress; Lanes 8 and 9, 2.5 μgm mitochondria from stressed animals. Scanning densitometry showed no significant differences between lanes 2-9.

Summary:

Pyridostigmine bromide (PB) was used repeatedly by personnel in the Gulf War to block the toxic effects of possible sarin exposure. PB is a reversible acetylcholine esterase inhibitor, which in presence of other chemical agents is thought to induce the symptoms of the Gulf War syndrome (see earlier discussion). In order to determine the role of the agents in development of the syndrome, we investigated the effects of PB in the presence of noise stress on brain stem mitochondrial energy coupling and electron transfer reactions.

PB treatment of rats in the presence of noise stress had little or no effect on mitochondrial energy coupling reactions, as shown using two different substrates, succinate and pyruvate/malate. This suggests that PB does not compromise the integrity of mitochondrial inner membrane of brain stem cells and that ATP synthase is fully able to synthesize ATP for vital cell processes. It is unlikely that PB, at the dose used, affects rat brain stem mitochondrial energy conservation.

Mitochondrial functioning can also be impaired by the inhibition of the energy conserving electron transfer reactions at specific sites. Specific assays for electron transfer were performed

on mitochondria isolated from brain stems of PB treated rats that underwent noise stress. Assays for cytochrome oxidase, succinate dehydrogenase, NADH dehydrogenase, and NADH-cytochrome c oxidoreductase activities were measured, and mitochondria from PB plus stress-treated animals exhibited approximately the same electron transfer activities as mitochondria isolated from control animals. This suggests that PB does not induce any site-specific effects on mitochondrial electron transfer reactions that could account for long-term depletion of cellular ATP stores. As mentioned previously, long-term depletion of cellular ATP could account for some of symptoms observed in Gulf War Syndrome patients.

The process of apoptosis in these treated animals was monitored by measuring endogenous cytochrome *c* content in our mitochondrial preparations. Our results show that PB treatment of rats did not affect the cytochrome *c* content of our isolated mitochondria. Also, the yield of mitochondria isolated was the same from all treated animals, suggesting no major mitochondrial damage occurred. These results suggest that PB plus stress did not cause a change in brain stem tissue at the cellular or sub cellular level.

Our conclusions from the PB plus stress experiments are that the agents did not affect brain stem energy metabolism as assessed by mitochondrial functioning and that PB plus stress did not induce significant apoptosis of brain stem cells. Therefore, it is unlikely that PB treatment at the concentration tested in these experiments can account for the symptoms described in the Gulf War Syndrome.

5. *Future Directions (July through October, 2002)*

We will focus on four areas for the next three months of the contract (See previous sections for a full discussion). Currently, we are assessing the effects of treatment of stressed animals with PB and DEET on mitochondrial activities and endogenous cytochrome *c* content. Second, we will submit the DEET data for publication in a peer-reviewed journal. Third, we will initiate studies on sarin starting with a dose response curve for the agent. This will be followed by sarin treatment (with and without stress) during the next three-month period of the contract. Finally, we will perform a positive control experiment in which the agent shows effects on the auditory brain stem response of the rat, cellular energy metabolism of brain tissue, and brain stem mitochondrial energy coupling and brain stem cell apoptosis. We see this as a critical control experiment. The agents that we have discussed using and that cause measurable effects are cis-platin and trimethytin.

Project 3: The study of enzymes involved in chemical metabolism, activity of dehydrogenases and esterase in human tissues with the goal of establishing whether there are alterations in populations characterized as chemically sensitive.

PI: Gerald M. Alter, Ph.D.

Statement of Work:

Specific goals for the second year of work included:

- Hiring and training a laboratory technician
- Determining which human samples are most useful for evaluating the activities of interest
- Establishing reliable assays for measuring desired activities in blood fractions.
- Establishing reliable activity values for various activities in control groups.
- Determining the best anticoagulation method to be used while collecting blood.
- Determining desired enzyme activity levels in plasma of a small test population.

Technician:

A technician was hired during the last year. This individual has direct responsibility for determining enzyme activities and performing other blood and protein fractionation duties relevant to this project. The technician's training for her current duties is complete.

Relevant samples:

During the previous year we have determined that insufficient levels of enzyme activities are found in cheek scrapings and saliva to warrant continued collection and analysis of these samples. Therefore these preparations will no longer be examined. During the first year of this project we similarly determined that hair follicle samples were not of sufficient size to be useful for enzymatic analysis.

Reliable Assay Methods:

During the past year we have not been able to solve difficulties with our SNAFL -based assay for paraoxonase and aryl esterase activity. These assays measure the changes in proton concentration that accompany paraoxon (paraoxonase) and phenyl acetate (aryl esterase) hydrolysis. These hydrolyses are catalyzed by the indicated respective enzymes. We believe that much of the problem is related to an affinity of SNAFL for unknown blood components. For this reason, another pH indicator dye, phenol red, has been tested. Though theoretically not as sensitive as SNAFL, phenol red is adequate for measuring plasma paraoxonase and aryl esterase activities. In addition, the assay is less sensitive to variations in protein content than the SNAFL-based assay, and therefore more easily calibrated. The more straightforward interpretation of phenol red time course is likely owing to absorbance rather than fluorescence emission intensity being used to monitor proton concentration changes. Specific assay conditions for proton-based assays are: 2 mM HEPES (pH 8.0), 2 mM $CaCl_2$, phenol red, 10 uM and about 10 μl of blood preparation in a 1 ml assay. When paraoxonase is measured, the assay solution is made 1.2 mM in paraoxon, and 2.63 M in NaCl. When aryl esterase is measured, the substrate, phenyl acetate is made 3.5 mM in the assay cuvette. In both assays absorbance changes are measured at 557 nm in a Cary 30 Spectrophotometer.

Assays for χADH, and aldehyde dehydrogenase continued to be developed during the year. Our current assay conditions for ALDH are: 50 mM TRIS (pH 8.0), 3 mM pyrazole, 1 mM $K_3Fe(CN)_6$, 5 mM NaCN and 10 μl of blood preparation in a 1 ml assay. Substrate concentrations are routinely 1.25 mM NAD and 0.8 mM formaldehyde. For χADH, normal assay conditions are 50 mM TRIS, (pH 8.0), 3.0 mM pyrazole, and about 20 μl of blood product in a 1ml assay. Substrate concentrations normally are: 1.25 mM NAD, 0.8 mM GSH, and 0.8 mM formaldehyde. Production of NADH during both these assays is measured spectrophotometrically at 350 nm.

Owing both to delays in preparation of an agent surety facility at WSU and to delays in obtaining the necessary monitoring equipment, we have not been able to test or develop proposed assay methods for the sarinase activity proposed in this study. Since the same samples are used for all assays, there will be an emphasis to get the sarinase assays functional, so that fresh samples can be used for all assays.

Control group activities:

Control samples from Anormal@ individuals (not chemically sensitive nor GW veterans) in Dayton, and in Dallas (also non-allergenic) were collected. Plasma activities from these controls were determined using our best enzymatic analyses. Results are summarized in the scatter graphs of Figures 3-1 thru 3-4. These figures demonstrate that appreciable scatter occurs among activity values of individuals in each control group. However, except for χADH activity, which seems to demonstrate more scatter among Texas controls than among Dayton controls, both groups have similar activity values and ranges.

Anticoagulant dependence of control activities:

A surprising observation was that activities of some controls were EDTA dependent. Among these are aryl esterase and paraoxonase activities (Figures 3-3 and 3-4). Supplementing EDTA (5 mM) - treated fractions with Zn2+ or Ca2+did not increase enzymatic activities. This is of particular interest since blood that was used in these studies was collected with EDTA as an anticoagulant. Therefore, it is possible that enzyme activities we are monitoring are perturbed in the collection process. (Complications associated with use of anticoagulants was previously mentioned by Michael Mackness ((1998) Atherosclerosis 136, 195-196)). To assess this possibility, the effect of EDTA, on serum collected from control individuals, was examined. Figure (3-5a and b) demonstrates that EDTA has a profound effect on serum aryl esterase activities. Most controls= χADH activity is also reduced. On the other hand, little if any effect on paraoxonase activity and no statistically significant perturbation of aldehyde dehydrogenase activity were observed. On the basis of these results we conclude that plasma activity measurements of aryl esterase activity and χADH are strongly perturbed when EDTA anticoagulant is used.

In fact, two aryl esterase activities that may be important to this study have been reported, one (EDTA insensitive) is serum albumin and the other=s is EDTA dependent. Either or both of these activities may be abnormal in chemically sensitive individuals, and will be measured using the EDTA sensitivity. Since activities of aryl esterase isolated from red or white blood cells of EDTA treated blood show essentially the same sensitivity to EDTA and serum enzymes, we conclude that the contents of red and white blood cells are free of EDTA even when this

compound is used as an anticoagulant. Activities of paraoxonase, aldehyde dehydrogenase, and χADH from white and red cell preparations also support this conclusion.

Though heparin anticoagulant perturbs the various activities we study less than EDTA, it does modify at least some of them. For this reason we conclude that serum is the best preparation to follow extra cellular activities. We further conclude that either EDTA, or heparin anticoagulants are useful when preparing cells whose enzymatic contents will be examined in this study.

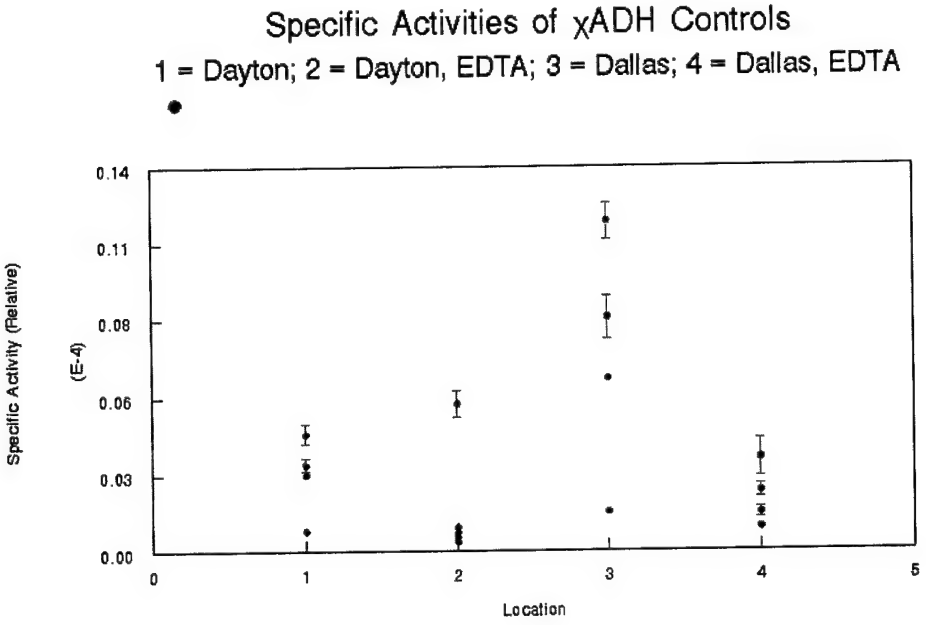


Figure 3-1.

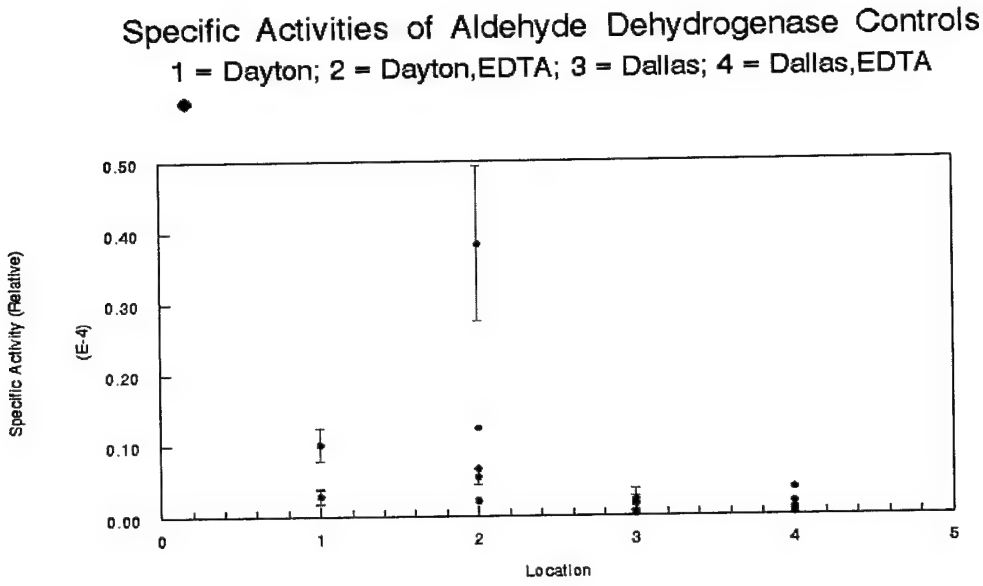

Specific Activities of Aldehyde Dehydrogenase Controls
1 = Dayton; 2 = Dayton,EDTA; 3 = Dallas; 4 = Dallas,EDTA
Specific Activity (Relative)
(E-4)
0.50
0.40
0.30
0.20
0.10
0.00
0
1
2
3
4
5
Location

Figure 3-2.

Figure 3-3.

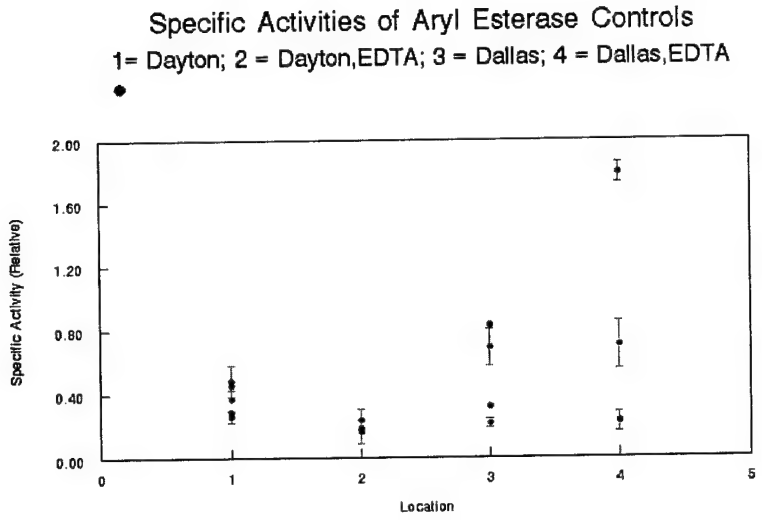
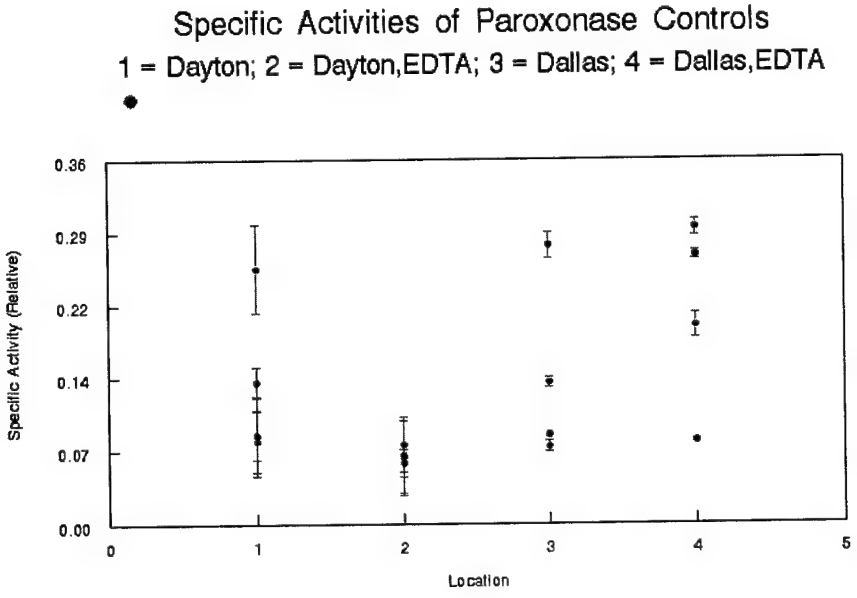


Figure 3-4.



Enzyme activity levels in Control and Test Groups:

Relative specific activities of control group blood fractions are summarized in Table 3-1. Each average specific activity represents at least 4 individuals. Relatively high standard deviations reflect variation in enzyme activity levels among individuals rather than the precision of measurements for each individual=s activities. Values for serum, white blood cells (WBC), and red blood cells (RBC) are currently being determined. Preliminary results indicate that ALDH activities are too low to measure in control group WBC preparations, despite concentrating the WBC contents, and so may not be examined further in this study.

Scatter graphs in Figures 3-6 thru 3-9 compare the activities of various enzyme activities in plasma of control and test group subjects. Though the plasma was collected with EDTA as an anticoagulant, all samples were collected and processed in the same way, so effects of EDTA on control and test groups should be similar. Yet, differences between control and test groups are apparent for at least χADH and ALDH activities (Figures 3-6 and 3-7) . In both cases, test group activities are appreciably higher than either Dayton or Dallas controls. The average and range of aryl esterase activity values are similar while paraoxonase activities may be slightly higher in test subjects relative to control populations (Figure 3-8 and 3-9). At least two possibilities could explain these results. First, if plasma preparations in the test group (from Texas) are contaminated by the contents of RBCs (rich in χADH (Sanghani, P., and coworkers (2000) Biochemistry 39, 10720-10729)) and/or WBC=s (a source tissue for ALDH isozymes as well as for χADH (Klyosov, A., Rashkovetsky, L., et. al. (1996) Biochemistry 35, 4445-4456)) these results might be expected. Second, if chemically sensitive individuals have abnormally induced ALDH and χADH activities the observed results would be expected. These possibilities will be distinguished using serum preparations. Similar activity analyses will be performed on WBC and RBC preparations control and test individuals.

Future Studies:

Future studies will primarily focus on analyses of blood fractions of patient blood. Fractions will include serum, WBC, and RBC. The demographic characteristics of new participants will be determined based on analysis of plasma, WBC, and RBC preparations from samples collected for current studies.

Table 3-1.

Relative Specific Activities

Activity		Average	Standard Deviation
PLASMA	Dayton control group		
Aryl Esterase		0.35695	0.0987
EDTA		0.19945	0.038266
Paraoxonase		0.1957	0.072533
EDTA		0.07215	0.007636
ALDH		6.26E-05	7.92E-05
EDTA		1.30E-05	1.46E-05
xADH		5.20E-06	5.62E-06
EDTA		1.51E-06	2.17E-06
PLASMA	Texas control group		
Aryl Esterase		0.52285	0.293127
EDTA		0.46445	0.739263
Paraoxonase		0.18115	0.091431
EDTA		0.2786	0.094322
ALDH		1.22E-06	9.12E-07
EDTA		2.01E-06	1.51E-06
xADH		7.03E-06	4.43E-06
EDTA		2.77E-07	3.60E-07
RBC	Dayton control group		
Xadh		3.57E-05	6.23E-06
EDTA		2.59E-05	3.13E-06

Figure 3-5.

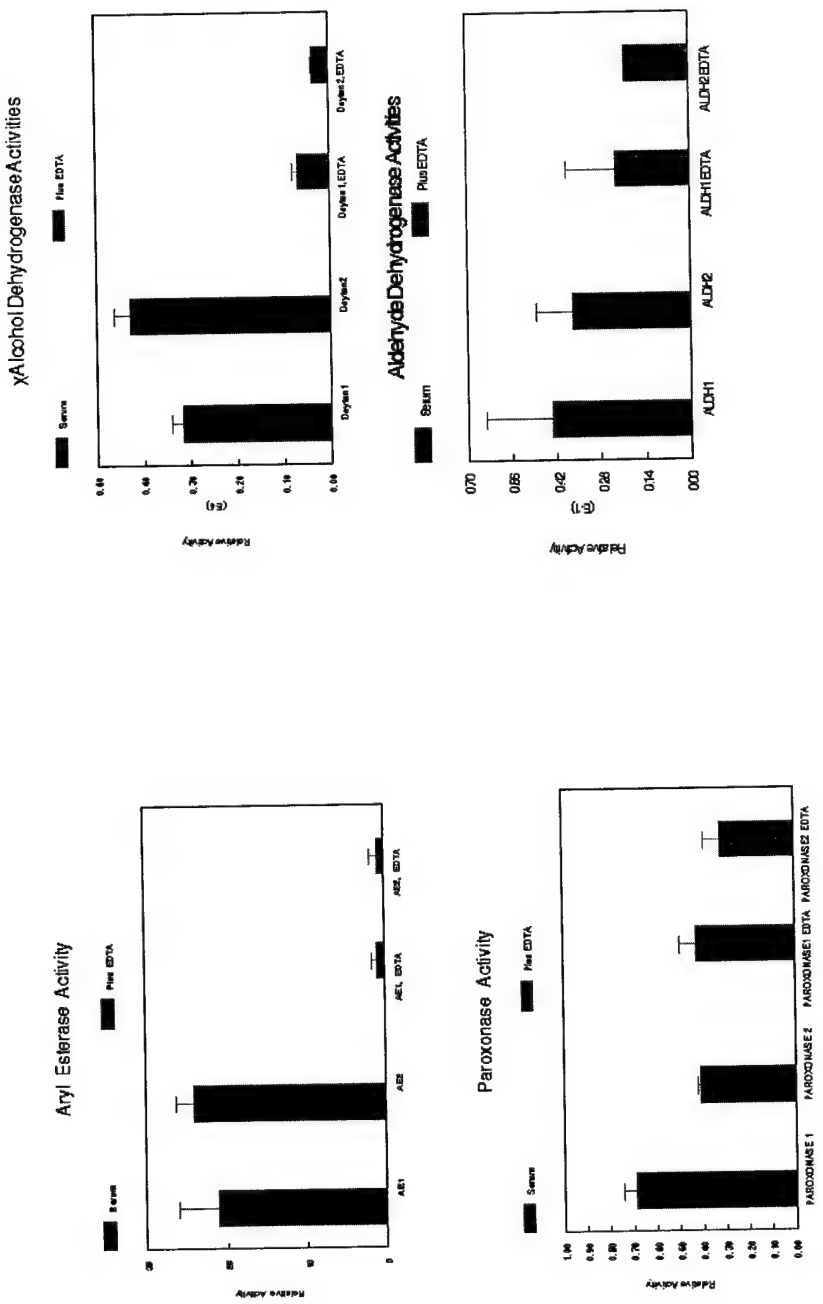

xAlcohol Dehydrogenase Activities
Serum
Plus EDTA
Dxylan1
Dxylan2
Dxylan1,EDTA
Dxylan2,EDTA
Relative Activity
(E4)
Aldehyde Dehydrogenase Activities
Plus EDTA
ALDH1
ALDH2
ALDH1EDTA
ALDH2EDTA
(E-1)
Aryl Esterase Activity
Serum
Plus EDTA
AE1
AE2
AE1, EDTA
AE2, EDTA
Relative Activity
Paroxonase Activity
Serum
Plus EDTA
PARAOXONASE 1
PARAOXONASE 2
PARAOXONASE1 EDTA
PARAOXONASE2 EDTA
Relative Activity

Figure 3-6.

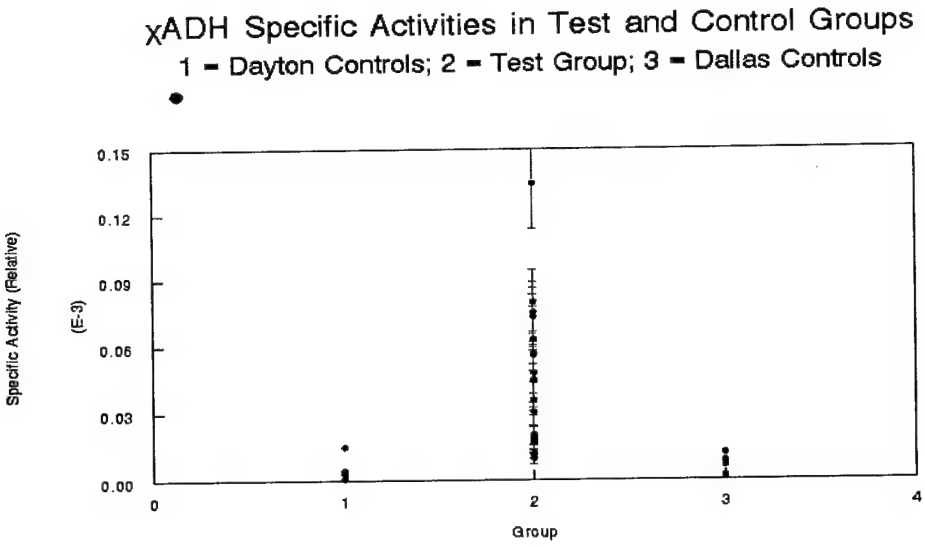


Figure 3-7.

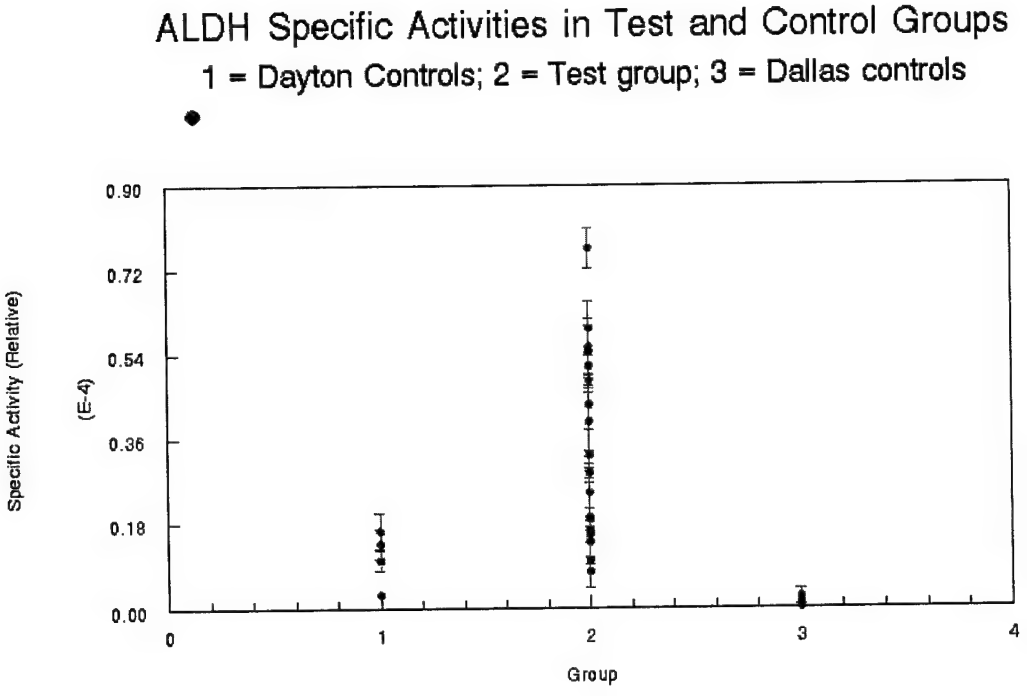


Figure 3-8.

Aryl Esterase Specific Activities in Test and Control Groups

1= Dayton Controls; 2 = Test Group; 3 = Dallas Controls

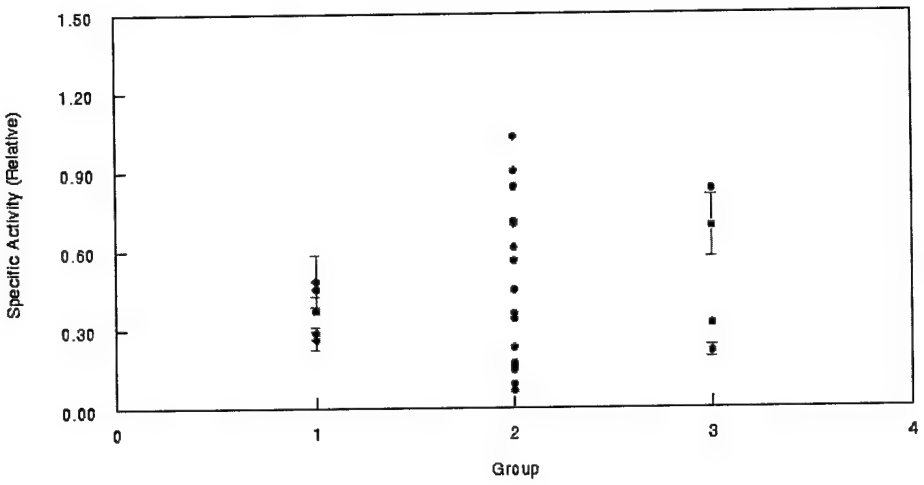
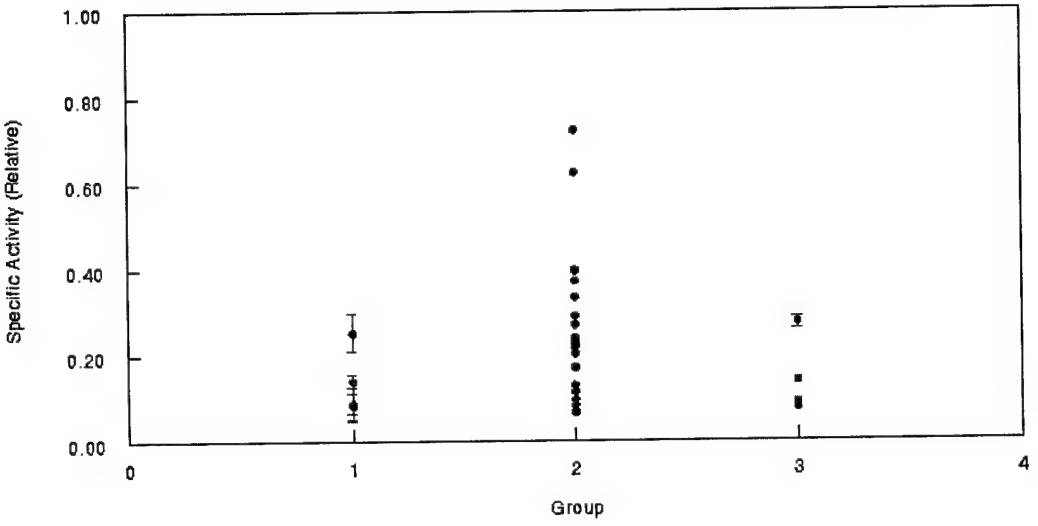


Figure 3-9.

Paroxonase Specific Activities in Test and Control Groups

1 = Dayton Controls; 2 = Test Group; 3 = Dallas Controls



Project 4: The study of gene expression using a DNA microarray system to test the effect of chemical exposure on a neuronal cell line.

Investigators: Steven J. Berberich, Ph.D., John Paietta, Ph.D. and Madhavi Kadakia, Ph.D.

STATEMENT OF WORK: YEAR 2

1. Analyzed gene expression profile data for PB treatment of HCN-2 cultured neurons (completed).
2. Differentiation of SHSY5Y neuroblastoma cells (completed).
3. Analyzed gene expression profile data for PB treatment of SHSY5Y cultured neurons (completed).
4. Personnel training, personnel medical examinations and testing of sarin effects on neuronal cells in culture (partially completed).
5. Gene Expression Profiles of sarin-treated cultured neurons (pending: sarin facility is not open).

SUMMARY PROGRESS REPORT

The focus of the work in year two has been on examining the effects of high (10^{-3}M) and low (10^{-8}M) doses of pyridostigmine bromide (PB) for 1-2 days on human neuronal cell lines (HCN-2 and SHSY5Y). The addition of SHSY5Y cells to the study was the result of two outcomes. First, there were few reproducible changes in gene expression observed with differentiated HCN-2 cortical neurons following 24 hour PB treatment, even when exposed to doses of PB ranging from 10^{-8}M to 10^{-3}M. Only a few genes showed reproducible changes of gene expression at longer PB treatments. We conclude from these findings that cortical neurons, lacking detectable AChE gene expression, are relatively unaffected at the gene expression level, to treatment with PB. The addition of the methodology to effectively differentiate SHSY5Y cells into human neurons gave us the opportunity to examine the effects of PB on a cultured neuronal cell that does express AChE. These experiments have clearly identified alterations at the level of gene expression when SHSY5Y cells are exposed to PB. The second reason for examining SHSY5Y cells was that the sarin facility, while completed and DOD approved, has not been open for sarin use until the monitoring and hood system are properly functioning. We anticipate starting our sarin treatments of neuronal cells in August, 2002. Lastly, we have made presentations at two conferences regarding our PB findings with HCN-2 cells, attended an User Group meeting held by Affymetrix and have undergone two searches to replace the original Research Assistant, Molly McGorry who left the laboratory in August 2001.

PRESENTATIONS (Abstracts in Appendix)

Chemical Warfare Agent: Toxicogenomics Conference, US Army Medical Research and Material Command, Maryland, November 2001, "Expression Profiling of Pyridostigmine Bromide responsive genes in Cultured Human (HCN-2) Cortical Neurons." Steven J. Berberich, Ph.D., Molly McGorry, John Paietta, Ph.D. and Madhavi Kadakia, Ph.D.

2002 EPA/ARFL Toxicology Meeting, Cincinnati, OH March 2002, "GeneChip Expression Profiling of Cultured Human Neurons exposed to Pyridostigmine Bromide" Steven J. Berberich, Ph.D., Molly McGorry, Phylip Chen, John Paietta, Ph.D. and Madhavi Kadakia, Ph.D.

DETAILED PROGRESS REPORT

4.1 PB treatment of HCN-2 cortical neurons

During this reporting period we performed data analysis on gene expression profiles of pyridostigmine bromide (PB) treated human neuronal HNC-2 cells. The data sets that were analyzed were PB doses ranging from 10^{-8}M to 10^{-3}M given for 24 to 48 hours. Attempts to examine the effects of PB beyond 48 hours of treatment were hindered by the observation that the differentiation state of HCN-2 cells at 24 and 48 hours did not match that seen with 72 hours (data not shown). An overview of the findings is provided in Table 4-1.

Table 4-1: RNA profiling results
PB treatment of human HCN-2 cells

PB dose	24 hrs.			48 hrs.		
10^{-3}M	N=9	Inc.	Dec.	N=12	Inc.	Dec.
	DC	0	6	DC	11	42
	MW	-	-	MW	3	27
	FC	-	-	FC	-	20
10^{-4}M	N=9	Inc.	Dec.	N=6	Inc.	Dec.
	DC	5	1	DC	146	214
	MW	-	-	MW	66	98
	FC	-	-	FC	24	43
10^{-8}M	N=9	Inc.	Dec.			
	DC	3	6	ND		
	MW	-	-			
	FC	-	-			

Table 4-1. N= number of comparative analyses run for a given dose and time. DC= number of genes that show an increase or decrease in over 50% of the comparison analyses. MW= number of gene that met the DC criteria AND showed a significant change in Ave. Diff. Using the Mann Whitney Test. FC= number of genes that met the MW criteria AND showed an Average Fold Changes greater or less than 2 fold. (-) represents that none of the 12,865 genes met that particular criteria. ND= no data. These gene expression profiles were performed using Hu95Aver2.0 chips and analyzed using DMT 2.0.

First, there are no significant changes in gene expression after 24 hours of PB treatment within the range of PB doses examined in this study. Interestingly, statistically significant changes of gene expression did emerge after 48 hours of PB treatment. A few of the interesting genes modulated at 48 hours of treatment with 10^{-4}M PB are listed in Table 4-2. Finally, there was no significant correlation seen at 48 hours between 10^{-3}M PB and 10^{-4}M PB. Based on these studies we conclude that HNC-2 cells, that do not express AChE, the identified target for both PB and sarin, show no significant alteration of gene expression when treated for short periods of PB (24 hours). The changes seen at 48 hours of PB treatment were reproducible. Interestingly, the genes changed at 10^{-4}M did not match those that changed at 10^{-3}M (for both increases and decreases). While it is possible that the changes observed are dose dependent, we anticipated seeing at lease a few genes changing within the 10-fold dose range examined. Therefore we will

be attempting to confirm these gene alterations using real time PCR. The Gene Expression Laboratory shares an ABI5700 with members of the Biochemistry and Molecular Biology Department. In two other funded projects, the GEL has developed the necessary technical expertise with RT-PCR to examine the genes listed in Table 4-2 to confirm that they are regulated in a dose-specific manner.

Table 4-2: Genes with altered expression following 48 hours of 10^{-4}M PB.

Increases	**Fold Change**	**Description**
CDK5 cyclin-dependent kinase 5, regulatory subunit 1 (p35)	2.4 +/- 0.7	The protein encoded by this gene is a neuron-specific activator of cyclin-dependent kinase 5 (CDK5), whose activation is required for proper development of the central nervous system. A truncated form of this protein is found to be accumulated in the brain neurons of patients with Alzheimer's disease. Accumulation of the truncated protein could lead to the deregulation of CDK5, and consequently create aberrantly phosphorylated forms of the microtubule-associated protein tau, which contributes to with Alzheimer's disease.
RBBP4 retinoblastoma binding protein 4	2.8 +/- 0.7	This gene encodes a ubiquitously expressed nuclear protein that belongs to a highly conserved subfamily of WD-repeat proteins. It is present in protein complexes involved in histone acetylation and chromatin assembly. It is part of the Mi-2 complex which has been implicated in chromatin remodeling and transcriptional repression associated with histone deacetylation. This encoded protein is also part of co-repressor complexes, which is an integral component of transcriptional silencing. It is found among several cellular proteins that bind directly to retinoblastoma protein to regulate cell proliferation. This protein also seems to be involved in transcriptional repression of E2F-responsive genes.
Decreases	**Fold Change**	**Description**
Placenta GF placental growth factor, vascular endothelial growth factor-related protein	-6.82 +/- 2.4	placental growth factor, vascular endothelial growth factor-related protein.
FLNA filamin A, alpha (actin binding protein 280)	-4.8 +/- 1	Filamin A, alpha (filamin 1); crosslinks actin filaments to membrane glycoproteins, transduces ligand-receptor binding to actin reorganization.
RAB3B member RAS oncogene family	-4.5 +/- 1.1	GTP-binding protein 3B; may be involved in vesicle transport; member of the RAB family of small GTPases.
Rap1B	-2 +/- 0.4	Ras related protein of unknown function.

Table 4-2. Fold change represents the increase or decrease in gene expression calculated from the average differences of PB-treated vs. control +/- the standard deviation. Descriptions of the gene products were obtained from Locus link using NetAffx.

4.2 Differentiation of SHSY5Y human neuroblastoma cells.

During this year, we tested methods to differentiate SHSY5Y cells, a human neuroblastoma cell line that has the capacity to differentiate into neuronal like cells (Encinas et. al., 2000). Briefly, SHSY5Y neuroblastoma cells were grown in F9/Eagle's media containing 10% fetal bovine serum. Differentiation was initiated by exposing the SHSY5Y cells to media containing to 10 μM all trans retinoic acid for 5 days. On day 6, the cells were treated with brain

derived neurotrophic factor in serum free media. Under these conditions, we were able to obtain a homogenous population of cells with neuronal morphology (Figure 4-1).

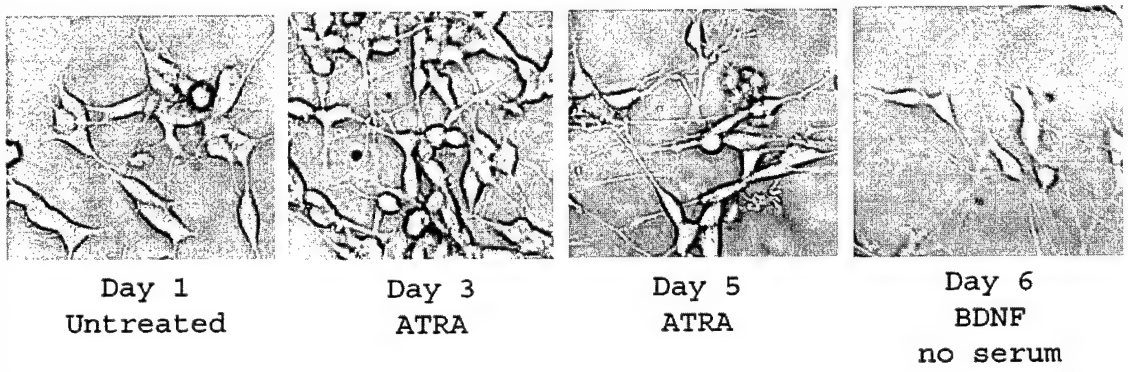


Figure 4-1: Morphological changes following differentiation of SHSY5Y cells treated for 5 days with retinoic acid followed by addition of BDNF in the absence of serum.

4.3 PB treatment of differentiated SHSY5Y neurons.

It was after day 6 of the differentiation regime that the differentiated SHSY5Y cells were mock exposed, exposed to 10^{-4}M PB or exposed to 10^{-3}M PB. Exposures were for either 24 or 48 hours and all were performed in media containing BDNF. Each treatment was performed in triplicate and used differentiated SHSY5Y cells. Total RNA was extracted 24 hours post treatment and processed for GeneChip experiments. Analysis was performed using the DMT 3.0 program.

Initially, genes that increased or decreased were identified for each PB treatment using the Mann Whitney test. Next, genes were classified as increasing, moderately increasing, showing no change, moderately decreasing or decreasing at each PB dose relative to no PB treatment. Those genes that increased or moderately increased in more than 50% of the comparative analyses were grouped. In a similar fashion, those genes that decreased or moderately decreased in more than 50% of the comparative analyses were also grouped. Lastly for each PB treatment the genes that increased in both screens (Mann-Whitney and Change) were grouped and a comparison was made between the two doses of PB. The results of this data mining approach are shown in the Venn diagrams in Figures 4-2 thru 4-4. The complete probe lists for each datamining approach can be obtained by contacting Dr. Steven Berberich, PI for Project 4.

The first grouping of genes in Figure 4-2 represent those genes that changed after 24 and 48 hours of the highest dose of pyridostigmine bromide (10^{-3}M). This particular dataset contains GeneChip data from 23 independent plates of differentiated SHSY5Y cells that were either mock treated (7), PB treated for 24 hours (6) or for 48 hours (7). A repeat of this experiment using another 18 independent plates of cells gave similar results (data not shown). Initially we compared the GeneChips from the 10^{-3}M PB treatment of SHY5Y neurons for 24 or 48 hours and detected that a few of the genes (those that increase or decrease based on both the Mann-Whitney and Change algorithms) showed identical gene expression patterns following 24 and 48 hours of 10^{-3}M PB treatment. Those genes are listed in Table 4-3.

Next, we performed datamining as described above on the datasets comparing 10^{-4}M PB treated neurons (24 and 48 hours) to the control, untreated cells. The Venn Diagrams representing the number of genes that showed decreases (A: 24 hrs; C: 48 hrs) and increases (B:

Figure 4-2: Venn Diagram of Gene Expression Changes in Neuronal SHSY5Y cells treated with 10^{-3}M PB for 24 or 48 hours.

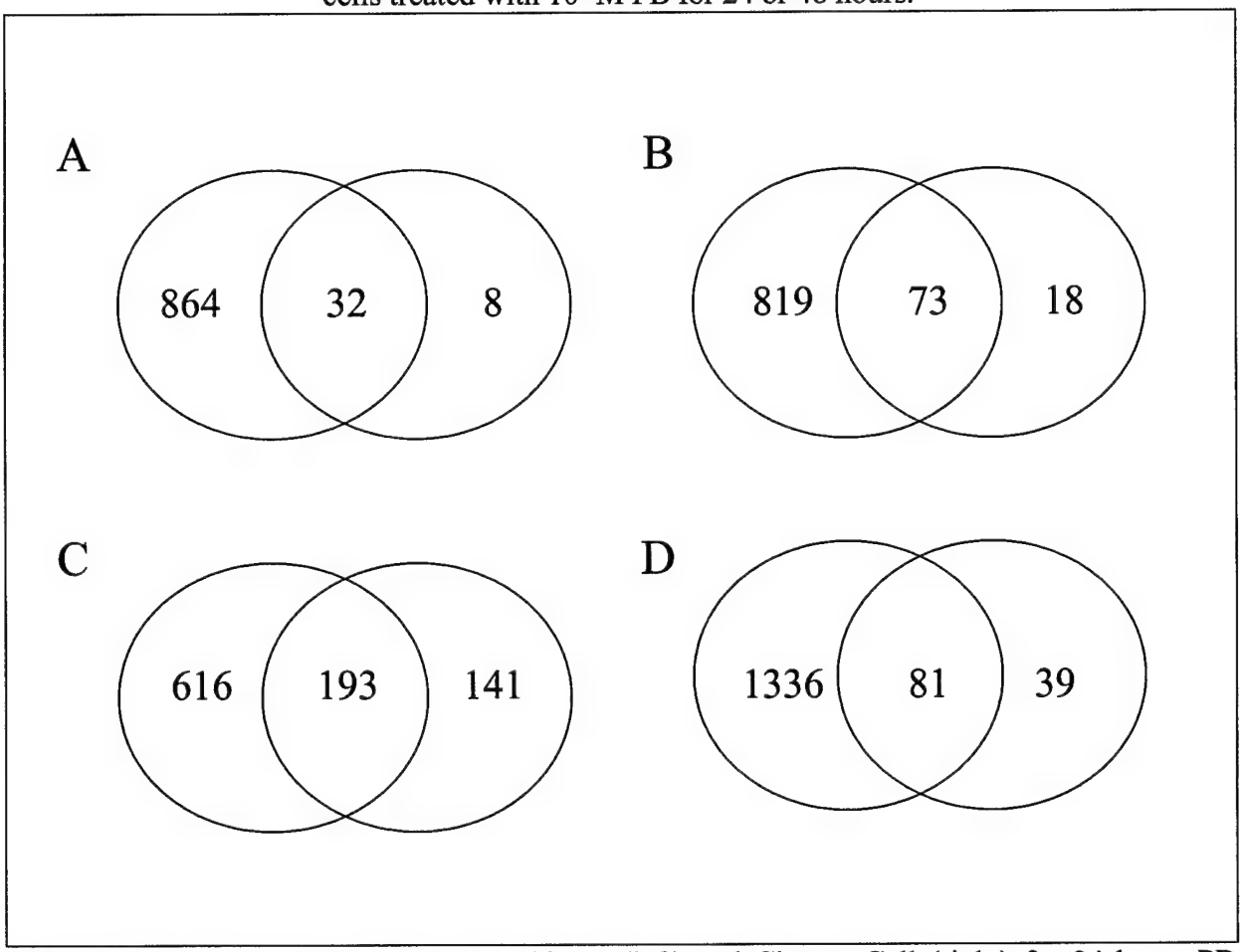


Figure 4-2: (A) Decreases by Mann Whitney (left) and Change Call (right) for 24 hours PB treatment. The genes common to both algorithms are shown in the center; (B) Increases by Mann Whitney (left) and Change Call (right) for 24 hours PB treatment. The genes common to both algorithms are shown in the center; (C) Decreases by Mann Whitney (left) and Change Call (right) for 48 hours PB treatment. The genes common to both algorithms are shown in the center; (D) Increases by Mann Whitney (left) and Change Call (right) for 48 hours PB treatment. The genes common to both algorithms are shown in the center.

Table 4-3: Genes with a consistent pattern of gene expression between 24 and 48 hours of 10^{-3}M PB treatment

GeneID	24 hr Change	24 hr Fold Change	48 hr Change	48 hr Fold Change	Description
1369_s_at	100% Dec	-1.42	81% Dec	-1.73	Interleukin 8
32818_at	77% Dec	-1.39	100% Dec	-2.53	hexabrachion (tenascin C, cytotactin)
35879_at	66% Dec	-1.3	75% Dec	-0.94	Pro-galanin
38125_at	88% Dec	-1.36	100% Dec	-2.1	Beta-migrating plasminogen activator inhibitor 1
38772_at	55% Dec	-1.28	100% Dec	-1.89	CYR61
39066_at	66% Inc	1.36	93% Inc	1.67	Microfibril-assoc. glycoprotein 4

Figure 4-3: Venn Diagram of Gene Expression Changes in Neuronal SHSY5Y cells treated with 10^{-4}M PB for 24 or 48 hours

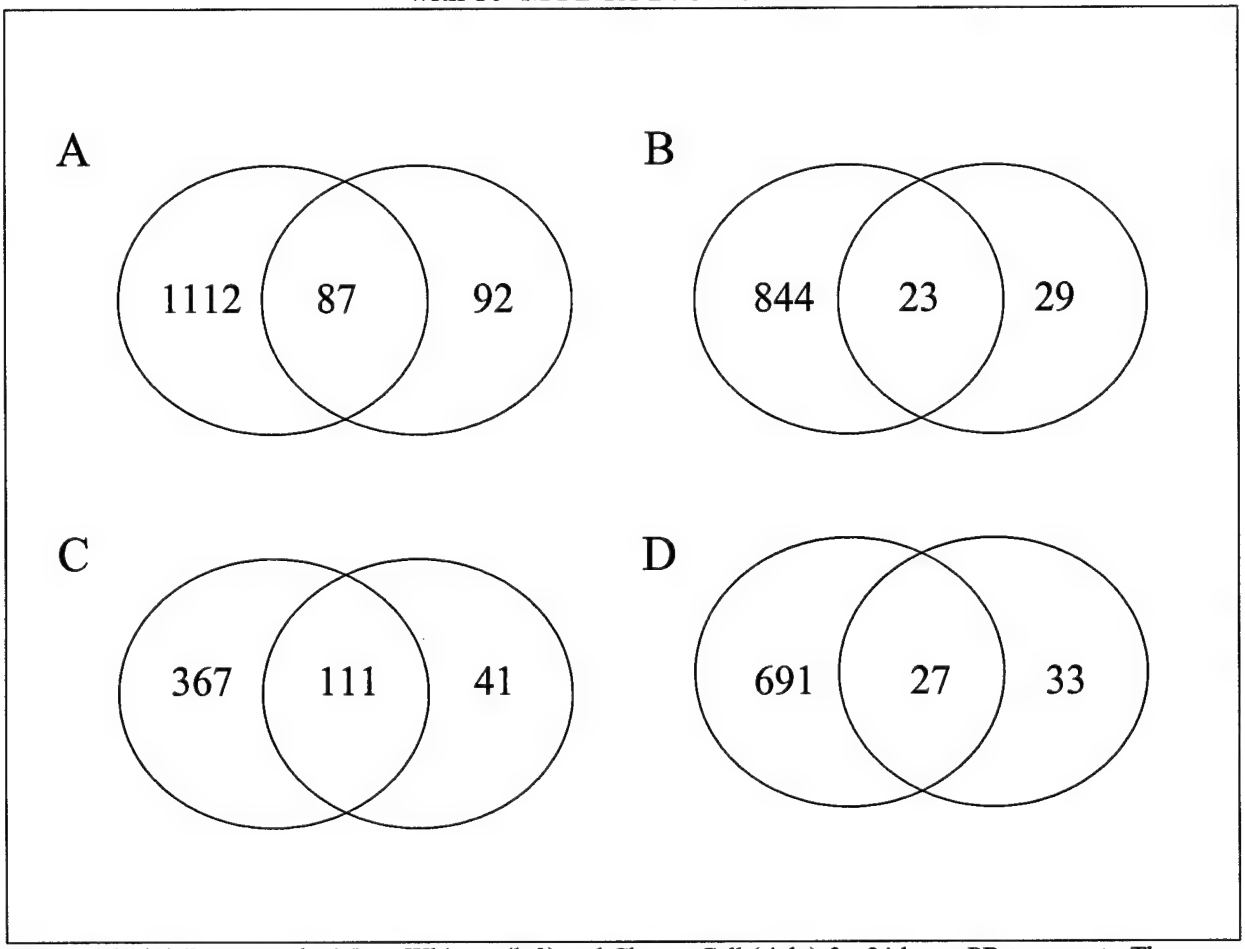


Figure 4-3: (A) Decreases by Mann Whitney (left) and Change Call (right) for 24 hours PB treatment. The genes common to both algorithms are shown in the center; (B) Increases by Mann Whitney (left) and Change Call (right) for 24 hours PB treatment. The genes common to both algorithms are shown in the center; (C) Decreases by Mann Whitney (left) and Change Call (right) for 48 hours PB treatment. The genes common to both algorithms are shown in the center; (D) Increases by Mann Whitney (left) and Change Call (right) for 48 hours PB treatment. The genes common to both algorithms are shown in the center.

24 hrs; D: 48 hrs) in gene expression using the Mann-Whitney (left circle) and Change algorithm (right circle) are shown in Figure 4-3.

Only a single gene was consistently decreased when comparing 24 hours of treatment at 10^{-4}M PB with 48 hours of treatment at the same dose (MTA1, metastasis associated gene, Fold change decrease –1.35; 100% decreased calls at 24 hours, 69% decreased calls at 48 hours). The lowest dose we have examined the effects of PB has been at 10^{-8}M for only 48 hours. Interestingly, there are sets of genes that are affected by PB treatment (Figure 4-4). Since we do not yet have 10^{-8}M treatment at 24 hours, we datamined the 48 hour treatment at the three different doses to ask whether there were any genes showing a consistent alteration of gene expression at all doses of PB attempted. Table 4-4 represents a Matrix Anaylsis that was used to compare the probe lists of common genes from each 48 hour treatment (the center number of genes in Figures 4-2 thru 4-4, panels C and D).

Figure 4-4: Venn Diagram of Gene Expression Changes in Neuronal SHSY5Y cells treated with 10^{-8}M PB for 48 hours

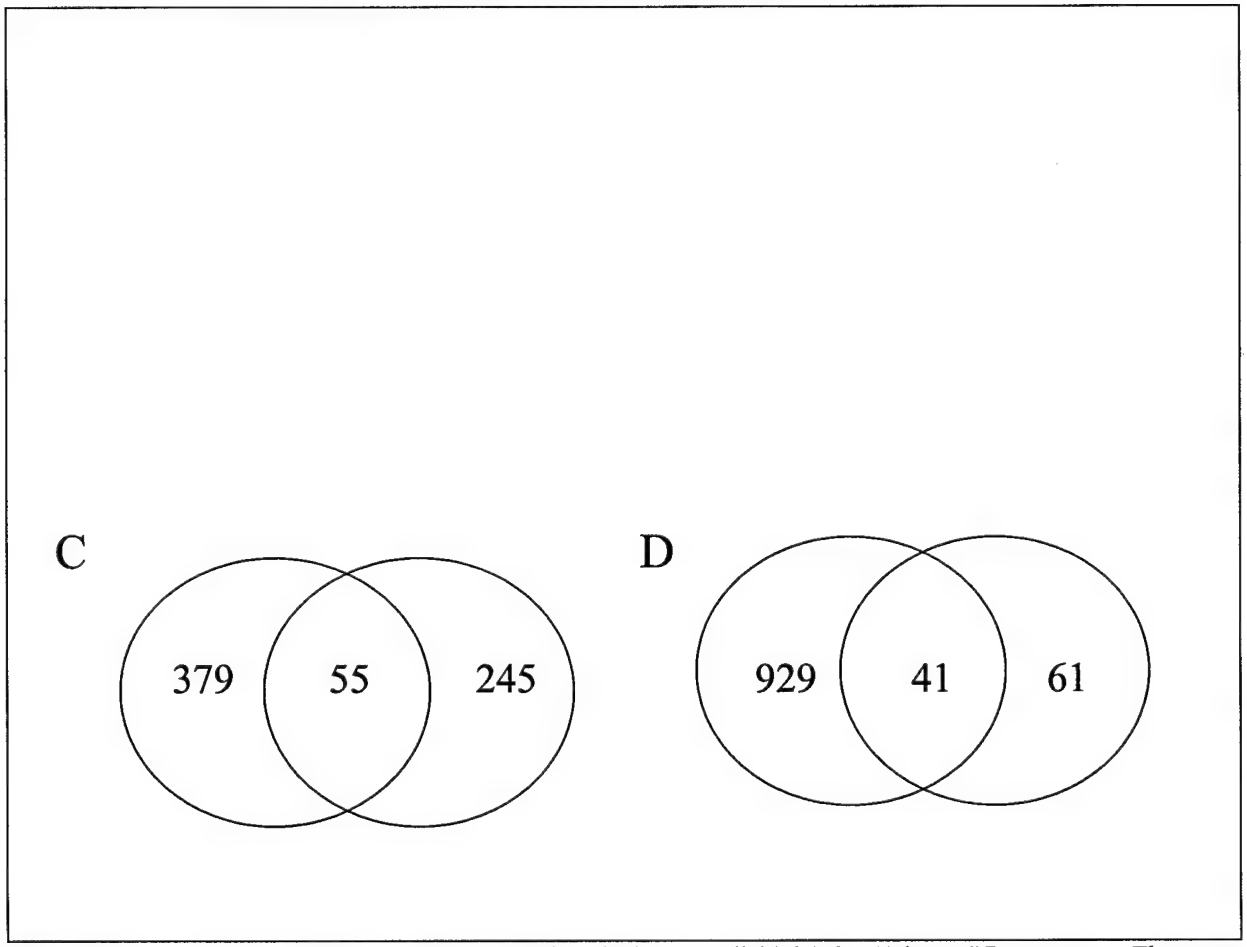


Figure 4-4: (C) Decreases by Mann Whitney (left) and Change Call (right) for 48 hours PB treatment. The genes common to both algorithms are shown in the center; (D) Increases by Mann Whitney (left) and Change Call (right) for 48 hours PB treatment. The genes common to both algorithms are shown in the center.

Table 4-4: Matrix Analysis to Identify Gene Lists with Common Genes

Probe List #1	Probe List #2	Inc. or Dec. Probe Lists	Score
48hr: 10^{-3}M	48hr: 10^{-4}M	Decrease	24.7
48hr: 10^{-3}M	48hr: 10^{-8}M	Decrease	11.3
48hr: 10^{-4}M	48hr: 10^{-8}M	Decrease	16.4
48hr: 10^{-3}M	48hr: 10^{-4}M	Increase	34.8
48hr: 10^{-3}M	48hr: 10^{-8}M	Increase	47.5
48hr: 10^{-4}M	48hr: 10^{-8}M	Increase	28.5

Table 4-4: A score of greater than 3 means that there is a less than 1:1000 chance that the overlap of genes detected when comparing probe list #1 to probe list #2 would occur by chance.

Matrix analysis enables you to compare probe lists (that include probe sets or spot probes) and determine the overlap between two probe lists (the probe sets or spot probes common to both lists). The matrix algorithm computes the probability (P-value) that the observed overlap is expected due to random chance. The algorithm converts the P-value to an overlap significance value that is displayed in the matrix. The overlap significance value = - logP, and may range from near zero to a large number. The results of the Matrix analysis demonstrates what we have observed by visually comparing the probes lists, that there is a set of consistent genes that are affected by PB at high and low doses (Table 4-4). We see a similar trend with the 24 hour data, comparing 10^{-3}M with 10^{-4}M (data not shown). When we complete the 10^{-8}M, 24 hours experiments, we will generate a list from these genes. The lists of genes that are seen increasing and decreasing at 48 hours at all PB doses tests are shown in Tables 4-5 and 4-6. So while only a few gene patterns emerged when comparing the 24 and 48 hours PB treated GeneChips (Table 4-3 and above), there were a significant number of genes that show similar patterns when treated with millimolar down to micromolar concentrations of PB. We intend to confirm a subset of these genes using Real Time PCR as described in section 4.2.

Table 4-5: List of Genes that are repressed at all doses of PB (48 hours).

10-3M Fold Change	10-4M Fold Change	10-8M Fold Change	Description
-1.19	-1.21	-1.35	Nucleosome assembly protein 1-like 1
-1.55	-1.36	-1.34	collagen, type XVI, alpha 1
-1.36	-1.55	-1.78	EGF-like-domain, multiple 4
-1.24	-1.12	-1.18	Isocitrate dehydrogenase 3 (NAD+) beta
-1.22	-1.25	-1.24	ELAV (embryonic lethal, abnormal vision, Drosophila)-like (Hu antigen D)

Table 4-6: List of Genes that are induced at all doses of PB (48 hours).

10-3M Fold Change	10-4M Fold Change	10-8M Fold Change	Description
1.6	1.38	1.74	Pax8
1.45	1.36	1.6	synapsin I (SYN1)
2.31	2.25	3.03	UDP glycosyltransferase 2 family, polypeptide B15
1.83	1.6	1.65	serine protease inhibitor, Kunitz type 1
3.32	2.5	3.23	AL050065:Homo sapiens mRNA; cDNA UNKNOWN
1.75	1.91	2.43	Homo sapiens cDNA, 5 end /clone=IMAGE-588365
1.54	1.37	1.71	Homo sapiens mRNA for KIAA0514 protein
1.77	1.62	1.82	Homo sapiens mRNA for KIAA0841 protein
1.56	1.43	1.6	p126 (ST5)' suppressor of tumorgenicty
1.66	1.46	1.54	phorbolin-like protein MDS019
1.64	1.47	1.65	beta-adrenergic receptor kinase (ADRBK1)
1.71	1.51	1.73	AD024 protein
1.69	1.56	1.77	Homo sapiens cDNA: FLJ23482 fis, clone KAIA03142
1.53	1.32	1.68	myosin-binding protein C, slow-type

4.4 Personnel training, personnel medical examinations and testing of sarin effects on neuronal cells in culture

The University arranged training and medical examinations for the members of Project 4 who will be engaged in the experiments using sarin. The required gloves, sarin and equipment have been purchased, however the sarin facility is not yet open. While we await the facility opening for use, we have begun to examine how both cell lines withstand growing in sealed chambers. This will require that the cells be grown in nutrient media containing HEPES. We anticipate that this modification of the media will be needed on during the times of sarin dosing.

4.5 Gene Expression Profiles of sarin-treated cultured neurons

We anticipate beginning these experiments during the next quarter, prior to the completion of year two in November 2002. The sarin facility is still not open.

KEY RESEARCH ACCOMPLISHMENTS

Project 1: Investigation of the effect of combined chemical and stress exposure on behavioral, cardiovascular, endocrine and cholinergic function.

- Organized a group presentation at the 2002 Society of Toxicology Meeting. Faculty, staff and students from Project 1 presented five abstracts to document the progress on the group effort to study stress/chemical interactions. Specifically, we gave presentations on the establishment of the stress model and the study of PB/stress interactions.
- Organized a symposium which was held at WSU in May, 2002. The Earl H. Morris Symposium, "Stress: Adaptation vs. Disease" was a forum for stress researchers. The keynote speaker was Dr. Paul Plotsky with other speakers from University of Cincinnati, Ohio State University and WSU. A poster symposium included presenters from the DoD project.
- Dr. Mariana Morris visited US Army Medical Research Institute of Chemical Defense in Edgewood Arsenal to meet with DoD scientists with the purpose of discussing results and progress.
- Drs. Morris, Grubbs and McDougal gave presentations at the Biosciences Review.
- Presented abstracts at 4 meetings, Society of Toxicology, Experimental Biology, Biosciences Review, Earl H. Morris Symposium, "Stress: Adaptation vs. Disease." Submitted two papers for publication with 3 to be submitted within a month.
- Work was completed on characterization of the chronic stress model. Studies combined analysis of behavioral, cardiovascular, endocrine and central cholinergic changes. The results indicate that intermittent shaker stress provides a good model for chronic stress exposure in mice.
- Studies of the circadian variation in behavioral and cardiovascular responses suggest that there are time related changes in responsiveness. The greatest responses were seen in the light period (inactive phase). These results are relevant to human studies which show circadian variation in cardiovascular pathologies.
- Studies of PB exposure show that PB at doses lower than 10 mg/kg failed to evoke any behavioral changes in mice. PB administered at 10 mg/kg/day resulted in exaggerated ASR and inhibition of locomotor activity in the open field without any prolonged or delayed effects. This suggests that PB has central neural effects when given chronically to mice.
- Tested and validated the chronic stress/PB exposure model in mice from the perspective of effects on acetyl cholinesterase activity in blood and different brain regions.
- Developed the methods for Western blot analysis of AChE protein in hypothalamus or other tissues.
- Used proteomic mass spectrometric methods for the analysis of hypothalamus, anterior and posterior pituitary tissue from PB/stress experiments.
- Developed the methods for the real-time PCR analysis of AChE, GAPDH and other hypothalamic peptides.
- Initiated studies to assess the effect of PB and stress on apoptosis in brain.
- Initiated studies to assess the effect of stress and PB on muscarinic receptor density in brain.

- Setup the breeding colony for oxytocin gene deletion mice, including development of the PCR methods and establishment of a computerized data base.
- Initiated studies using a new genetic model, oxytocin knock out. Preliminary results show that there are prominent changes in the stress responses after deletion of the oxytocin gene.
- Set up the procedures for the use of the Surety facility for the use of CWA. This included the development of the plans for safety training, blood testing, experimental protocols and other issues.

Project 2: The effect of stress and chemical exposure on auditory brain responses, energy metabolism and tissue chemical constituents in an animal model.

- Completed the chronic low-dose study involving DEET treatment +/- noise stress.
- Completed the chronic low-dose study involving pyridostigmine bromide treatment + noise stress.
- Initiated the chronic low-dose study of drug synergism involving DEET + pyridostigmine bromide + noise stress.
- Quantitated the endogenous cytochrome c content of rat brainstem mitochondria using immunoblots.
- Optimized experimental procedures for NMR analyses of brainstem extracts in vitro.
- Constructed a new NMR probe for studies of rat leg muscle in vivo.
- Prepared and examined rat cochleas by scanning electron microscopy for evidence of hair cell loss/damage following noise-exposure.
- Optimized method for collection of pre- and post-noise-exposure blood samples for corticosterone analysis. Analyzed samples for elevated corticosterone levels as evidence of noise-induced stress.
- Hired and trained new laboratory personnel.
- Manuscript in preparation concerning the first two items.

Project 3: The study of enzymes involved in chemical metabolism, activity of dehydrogenases and esterase in human tissues with the goal of establishing whether there are alterations in populations characterized as chemically sensitive.

- Hired and trained a laboratory technician.
- Determined which human samples are most useful for evaluating the activities of interest.
- Established reliable assays for measuring desired activities in blood fractions.
- Established reliable activity values for various activities in control groups.
- Determined the best anticoagulation method to be used while collecting blood.
- Determined desired enzyme activity levels in plasma of a small test population.

Project 4: The study of gene expression using a DNA microarray system to test the effect of chemical exposure on a neuronal cell line.

- Analyzed gene expression profile data for PB treatment of HCN-2 cultured neurons.
- Differentiation of SHSY5Y neuroblastoma cells (completed).
- Analyzed gene expression profile data for PB treatment of SHSY5Y cultured neurons
- Personnel training, personnel medical examinations and testing of sarin effects on neuronal cells in culture.
- Gene Expression Profiles of sarin-treated cultured neurons (pending: sarin facility is not open).

REPRORTABLE OUTCOMES

Presentations:

McDougal, Jim. A workshop on "Dermal Risk Assessment – Practice and Controversy" at the "Theories and Practices in Toxicology and Risk Assessment Conference, Cincinnati, OH, 18 April 2002.

Ropp, S., Bernatova, I., Paton, S.J., Price, W.A., Morris, M. and Cool, D.R., SELDI-TOF mass spectrometry proteomic analysis of pyridostigmine Br and stress on the HP-axis. 41st Annual Meeting. March 17, 2002. Nashville, TN, Abs #1086. Poster-Discussion: Toxicologic Application of Proteomics- Discussion Panelist D. R. Cool.

Abstracts:

Project 1:

Bernatova I, Price WA, Grubbs RD, Morris M. Effect of chronic pyridostigmine bromide treatment on blood pressure and acetylcholinesterase activity in mice. The Toxicologist 66: 2002.

Bernatova, M. Dubovicky, M. Key, J.B. Lucot, M. Morris: Chronic stress alters cardiovascular and endocrine responses in mice. The FASEB Journal 16(4), A506, 2002.

Bernatova, M. Dubovicky, S.J. Paton, J.B. Lucot, M. Morris: Pyridostigmine treatment alters stress responsiveness in mice. Bioscience Review. US Army Medical Defense. Abstract Program Book. P123, 2002.

Dubovicky, M., I. Bernatova, M. Morris, J. B. Lucot: Development of a model for chronic stress exposure in mice. The Toxicologist 66 (1-S), 252, 2002.

Grubbs, RD, WA Price, BS Mauck, I Bernatova, SJ Paton, DR Cool, JB Lucot, M Morris (2002) "Cholinesterase Activity in Mice Chronically Exposed to Pyridostigmine Bromide." The Toxicologist 66 (1-S), 252, 2002.

Grubbs, RD, WA Price, BS Mauck, I Bernatova, SJ Paton, DR Cool, JB Lucot, M Morris (2002) "Cholinesterase Activity in Mice Chronically Exposed to Pyridostigmine Bromide", abstract/poster presentation at the Theories and Practices in Toxicology and Risk Assessment Conference in Cincinnati, OH, April 15-18, 2002.

Grubbs, RD, WA Price, BS Mauck, SA Ropp, I Bernatova, SJ Paton, M Morris, JB Lucot, DR Cool (2002) "Regional Differences in Brain Cholinesterase Activity and Protein Expression in Mice Following Subacute Stress and Exposure to Pyridostigmine Bromide." Bioscience Review. US Army Medical Defense. Abstract Program Book. P118, 2002.

Jackson, N., J.B. Lucot: Stress alters the motor and neurochemical responses to MPTP. Earl H. Morris Symposium, "Stress: Adaptation vs Disease," 2002.

Lucot, J.B., M. Dubovicky, J.R. Wells: Effect of pyridostigmine and chronic shaker stress on acoustic startle response, pre-pulse inhibition and open field behavior of mice. Earl H. Morris Symposium, "Stress: Adaptation vs Disease," 2002.

McDougal, J.N. Molecular Changes in Skin Following Acute Dermal Exposures to Irritating Chemicals. Bioscience Review. US Army Medical Defense. Abstract Program Book. P128, 2002.

Ropp, S., Bernatova, I., Paton, S.J., Price, W.A., Morris, M. and Cool, D.R., SELDI-TOF mass spectrometry proteomic analysis of pyridostigmine Br and stress on the HP-axis. The Toxicologist 66 (1-S), 1086, 2002.

Wells, J. R., M. Dubovicky, J.B. Lucot: Effects of shaker stress on the open field behavior of mice under diurnal and nocturnal conditions. International Stress *Symposium 2002.*

Project 2:

Bicknell, R., N. V. Reo, L. Prochaska, M. Forquer, L. Shroyer, A. Neuforth, and T. Young. "ABR, NMR, and energy metabolism studies of the effects of long-term exposure to low doses of DEET in the rat". Annual meeting of the Association for Research in Otolaryngology (ARO), St. Petersburg Beach, FL, February 2002.

Project 4:

Steven J. Berberich, Ph.D., Molly McGorry, John Paietta, Ph.D. and Madhavi Kadakia, Ph.D. Chemical Warfare Agent: Toxicogenomics Conference, US Army Medical Research and Material Command, Maryland, November 2001, "Expression Profiling of Pyridostigmine Bromide responsive genes in Cultured Human (HCN-2) Cortical Neurons."

Steven J. Berberich, Ph.D., Molly McGorry, Phylip Chen, John Paietta, Ph.D. and Madhavi Kadakia, Ph.D. 2002 EPA/ARFL Toxicology Meeting, Cincinnati, OH March 2002, "GeneChip Expression Profiling of Cultured Human Neurons exposed to Pyridostigmine Bromide"

Manuscripts:

Bernatova, I., M.P. Key, J.B. Lucot, M. Morris: Circadian Differences in Stress-Induced Pressor Reactivity in Mice, In Revision, Hypertension, 2002.

Bernatova, I., M. Dubovicky, J. B. Lucot, M. Morris: Effect of pyridostigmine treatment on behavioral and cardiovascular parameters in mice. in preparation for Pharmacology, Biochemistry & Behavior.

Cool, D.R. and D. DeBrosse, Comparison of preparative methods for analysis of peptide hormones by radioimmunoassay and SELDI-TOF MS. (Revisions submitted to J. Chromatography B, 2002).

Dubovicky, M., J.R. Wells, M. Morris, J. B. Lucot: Chronic shaker stress alters day/night behavioral patterns in mice. Submitted Physiology and Behavior, 2002.

Dubovicky, M., J.R. Wells, S. Paton, B. Mauck, M. Morris, J.B. Lucot: Effects of chronic shaker stress and pyridostigmine treatment on acoustic startle response, pre-pulse inhibition and open field behavior in mice. in preparation for Toxicological Sciences.

CONCLUSIONS

This is a comprehensive multidisciplinary project which involves nine principal investigators and four major projects. The contract has resulted in the development of a number of sophisticated research methods which will be used to address the issues related to CWA responsiveness and its modulation by stress. The approaches range from gene screening using the Affymetrics DNA microarray to the telemetric monitoring of behavior and cardiovascular parameters in conscious mice. Thus, there is much potential for contribution to the environmental sciences, particularly as related to the military.

The key accomplishment section clearly illustrates the progress which was made in the second year of the project. Many of the research projects have come to fruition, resulting in scientific presentations, publication of abstracts and submission of manuscripts. The net result is an expansion of our knowledge base as related to stress/chemical interactions, genomic and proteomic changes associated with PB exposure and enzymatic changes in human populations (control and chemically sensitive). During the third year of the project, the focus will be on the initiation of studies of low dose exposure to Sarin, completion of other projects, presentation at National Meetings and submission of publications.

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APPENDIX

Project 1:

Bernatova, I.[1,2], B Price[1], R Grubbs[1] and M Morris[1]. Effect of chronic pyridostigmine bromide treatment on blood pressure and acetylcholinesterase activity in mice. [1]Department of Pharmacology and Toxicology, Wright State University School of Medicine, Dayton, Ohio; [2]Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovak Republic.

Pyridostigmine bromide (PB) is used primarily for treatment of myasthenia gravis and for prophylactic protection against oranophosphorus nerve poisoning. PB acts by reversible inhibition of acelylcholinesterase (AChE) which is involved in the metabolism of ACh and thus in regulation of neuromuscular and autonomic function. However, there is a little information on the effect of PB on cardiovascular (CV) function. Experiments were performed to determine the time course of the effect of PB on plasma AChE and blood pressure (BP) and heart rate (HR) in conscious mice. The experiment used C57 male mice with chronic carotid arterial catheters for CV measurements and collection of blood samples. PB was administered sc using osmotic minipumps at 1 and 3 mg/kg/day for 7 days. The controls were sham-operated with minipumps filled with saline. BP and HR (sampling rate 85 Hz) were measured for 24 hr on day 0 and days 3 and 7 after minipump insertion with blood samples (25 μl) collected on the same days for AChE determination. Mean BP of the control group was 108±5 and 103±4 mm Hg during the dark and light period, respectively. HR was 499± 62 and 478±63 beats/min during the dark and light period, respectively. PB treatment had no effect on BP and HR. Basal AChE activity was 0.44±0.18 μmol/min/ml. PB treatment (1 mg/kg/day) had no influence on AChE activity. However, 3 mg/kg/day of PB decreased AChE activity on days 3 and 7 significantly by 48±29% and 84±16%, respectively. In conclusion, chronic low dose PB exposure in mice decreased AChE activity but had no effect on BP and HR.

Bernatova, I.,[1,2] M Key[1], M Morris[1]. Chronic stress alters cardiovascular and endocrine responses in mice. [1]Department of Pharmacology and Toxicology, Wright State University School of Medicine, Dayton, Ohio; [2]Institute of Normal and Pathological Physiology, SAS, Bratislava, Slovak Republic.

Abstract submitted to the Experimental Biology Meeting in New Orleans:

Studies were performed to evaluate the effect of stress on blood pressure (BP), heart rate (HR) and corticosterone (Cort) responses in conscious mice. C57Bl male mice with chronic carotid arterial catheters were exposed to intermittent shaker stress for 7 days with measurement of BP and HR. A comparison was made between the responses on day 1 (acute) and day 7 (chronic). Differences in the day/night stress responses were noted on day 1, but not on day 7. A 2 min stress on day 1 increased BP by 23% during the light period (1100 h) and 12% during the dark period (2300 h). The tachycardia was 19 % and 9 % during the light and dark periods, respectively. After each shaking session, BP and HR gradually returned to baseline. The time course of the stress responses was different on day 1 and 7 with a more rapid return to baseline values on day 7. A separate experiment determined the effect of acute and chronic stress on plasma Cort levels. There was a marked attenuation of the Cort response in the chronically stressed animals, an increase of 53.3±12 as compared to 118.3±18.5ng/ml. In conclusion, there is habituation of both cardiovascular and endocrine responses in chronically stressed mice.

Bernatova, I[1,2], Dubovicky M[1,3], Paton SJ[1], Lucot JB[1], Morris M.[1] Pyridostigmine treatment alters stress responsiveness in mice. [1]Department of Pharmacology and Toxicology, Wright State University School of Medicine, Dayton, Ohio; [2]Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovak Republic; [3]Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovak Republic.

Submitted to the Bioscience Meeting:

Pyridostigmine bromide (PB) was used for prophylactic protection against organophosphorus nerve poisoning in the Gulf War. Since there is evidence that stress enhances the effect of PB, we decided to study stress/chemical interactions in a mouse model. Using male C57Bl mice, we tested the effect of intermittent shaker stress on body weight (BW), food intake and drinking activity and corticosterone levels. Stress was delivered automatically for 2 min periods (150 cycles/min), 45 times/day for 7 days. Stress increased water intake, produced no change in food intake and significantly decreased BW (5% change). In a separate experiment, the effect of interactions of chronic stress with PB, acetylcholinesterase (AChE) inhibitor, on corticosterone levels was investigated. PB was delivered sc, using osmotic minipumps, at doses of 3 and 10 mg/kg/day. Stress significantly increased corticosterone levels (4-fold increase). PB treatment at the dose of 10 mg/kg/day blocked the corticosterone response to stress. This suggests that cholinergic systems are important in mediating stress reactivity. In conclusion, we have developed a model for chronic stress and chemical exposure in mice. This model combines emotional and physical stress with chemical toxicity, which may emulate stress conditions experienced by humans.

This work was supported by the U.S. Army Medical Research and Material Command under Contract DAMD17- 99214005.

Dubovicky, M.[1,2], I Bernatova[1,3], M Morris[1], J B Lucot[1]. Development of a model for chronic stress exposure in mice. [1]Dept Pharmacology/Toxicology, Wright State University, School of Medicine, Dayton, OH, USA; [2]Inst Exp Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia; [3]Inst Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovakia.
Sponsor: P G Gunasekar.

Abstract submitted to the Society of Toxicology Meeting, 2002

Chronic stress contributes to physical and mental illness and may interact with chemical toxins. Our previous experiments demonstrated that acute stress potentiates neurotoxicity (Lucot, 1999). Thus, we conducted controlled scientific studies of the responses to stress preparatory to studying the interactions of stress and chemical toxins. We have developed a unique model of chronic emotional stress in mice based on intermittent shaker stress. Mice are kept in their home cages fixed to a cage rack mounted on the shaker power unit. Standard pellet diet and tap water are provided ad lib. Our experimental paradigm enables the study of the neuroendocrine, cardiovascular and behavioral responses to acute as well as chronic stress. Chronic stress did not have any unfavorable effect on the health of the mice. There was 10 % of loss in body weight. However, after discontinuation of chronic stress the mean values of body weight returned to the values of non-stressed controls. Neuroendocrine and cardiovascular assessment revealed significant increases in plasma corticosterone, blood pressure and heart rate in acutely and chronically stressed mice. Chronic emotional stress did not have any effect on total open field activity or on elevated plus maze behavior. However, emotional reactivity on the elevated plus maze as measured by defecation rate increased in the chronically stressed mice 7 days after stress. Unlike the chronic stress, acute stress resulted in significant decreases in locomotor activity in the open field test. Our results show that the chronic intermittent shaker stress provides an excellent model for stress exposure and for use in testing stress/chemical interactions.
Supported by DoD, contract No. 99214005.

Grubbs, R.D., W A Price, B S Mauck, I Bernatova, S J Paton, D R Cool, J B Lucot, M Morris. Cholinesterase activity in mice chronically exposed to pyridostigmine bromide. Department of Pharmacology and Toxicology, Wright State University School of Medicine, Dayton, OH, USA.

Abstract submitted to the Society of Toxicology Meeting, 2002

Pyridostigmine bromide (PB), given to soldiers during the Persian Gulf War as a prophylactic against potential nerve gas attack, is now thought to be a possible causative agent of the symptoms of Gulf War Syndrome. We have developed a murine model to test the effect of chronic exposure to PB on cholinesterase (ChE) activity in the blood and the brain. C57B/J mice were surgically implanted with osmotic mini-pumps to deliver a steady dose of PB over 7 days. ChE activity was determined by a modified version of the colorimetric assay of Ellman, *et al.* Total ChE activity was measured in diluted whole blood and brain homogenate samples. Blood acetylcholinesterase (AChE) activity was determined by inhibiting butyrylcholinesterase (BChE) with iso-OMPA (tetraisopropylpyrophosphoramide). BChE activity was then calculated by subtracting AChE activity from total ChE activity. Following 7 days of exposure to PB, reductions of blood ChE activity of 27%, 42%, and 55% were seen in the 3, 6 and 10 mg/kg/D dosage groups, respectively, when compared to pretreatment levels. The decrease in blood ChE activity, due primarily to a drop in AChE activity, indicated that the osmotic mini-pump delivery system was functioning properly. We found no change in CHE activity in sham-implanted control animals. Interestingly, while analysis of prefrontal cortex showed no significant change in AChE activity after exposure to PB, we observed a dose-dependent decrease in AChE activity (48% at 3 mg/kg) in the hypothalami of PB treated mice (see Ropp, *et al.* abstract, this meeting). We are currently evaluating ChE activity in other brain regions of these animals and other mice exposed to both chronic stress and PB. Our findings indicate that chronic exposure to PB produces a dose-dependent decrease in blood and hypothalamic cholinesterase activity possibly contributing to symptoms of Gulf War Syndrome. This research is funded by DoD contract DAMD17-00-C-0020.

Grubbs, R.D., W A Price, B S Mauck, S A Ropp, I Bernatova, S J Paton, M Morris, J B Lucot, D R Cool. Regional Differences in Brain Cholinesterase Activity and Protein Expression in Mice Following Subacute Stress and Exposure to Pyridostigmine Bromide. Department of Pharmacology and Toxicology, Wright State University School of Medicine, Dayton, OH, USA.

Submitted to the Bioscience Meeting:

Pyridostigmine bromide (PB), given to soldiers as a prophylactic against potential CWA exposure, is thought to be a possible causative agent of the symptoms of Gulf War Syndrome. We have developed a murine model for subacute exposure to PB and stress in order to analyze their effects on specific regions of the brain. ChE enzyme levels and proteomic profiles (SELDI-TOF mass spectrometry) were measured in prefrontal cortex, brain stem, and hypothalamus after PB, stress (intermittent shaker stress for 7 days) and the combination. C57Bl mice were surgically implanted with osmotic mini-pumps to deliver a steady dose of PB over 7 days. Analysis of blood PB concentrations indicated that the osmotic mini-pump delivery system was functioning properly. ChE activity was determined by a modified version of the standard colorimetric assay. Following 7 days of exposure to PB, reductions of blood ChE activity of 17% and 29% were seen in the 3 and 10 mg/kg/D dosage groups, respectively, when compared to sham control levels. Interestingly, a significant dose-dependent decrease in hypothalamic AChE activity (>40% at 3 mg/kg/day) was observed in the PB -treated animals, while analysis of prefrontal cortex showed no change. Adding stress to this paradigm produced a statistically significant decrease in CHE activity in the prefrontal cortex. In the brain stem, PB exposure produced a dose-dependent increase in AChE activity that was reversed by concomitant stress exposure. Proteomic analysis of the same stress and PB-treated hypothalamic homogenates by SELDI-TOF MS showed significant and similar changes in the hypothalamic peptide/protein profiles for both stressed and PB-treated mice. Ratio analysis of the protein profiles indicated a decrease in peak intensity for both proteins (>4000 d) and peptides (<4000 d). However, the decrease in the ratio of peak intensity was greater for proteins (2.0-7.5) than for peptides (1.3-2.0), suggesting a buildup of peptides within the cells. Our findings indicate that subacute exposure to PB and stress produces dose-dependent changes in brain ChE activity that vary depending on area analyzed. In the hypothalamus, our results suggest that stress and PB both affect the peptide protein profiles in the hypothalamus by changing either the protein expression or peptide processing and secretion patterns of the cells. This work was supported by the U.S. Army Medical Research and Materiel Command under Contract DAMD17-00-C-0020.

Jackson, N. and J.B. Lucot. Stress alters the motor and neurochemical responses to MPTP. Dept. Pharmacology, Wright State University, Dayton, OH 45435.

Investigations of the origin of Gulf War Syndrome have uncovered stress as a factor in the response to toxins. The present study investigated the effect of shaker stress on the dopaminergic toxin, MPTP in C57Bl mice. This psychological stressor was produced by horizontal displacement of 3.8 cm at 2.5Hz (150 CPM) intermittently for 4 hours. MPTP was administered 10 min before stress.

When DDC was given 20 min before MPTP, stress changed the locomotor motor activity response from depression to stimulation. Stress had no effect on dopamine damage in the caudate or accumbens but potentiated it in the amygdala, which was the area most sensitive to deficits. Without DDC, stress reduced the motor deficits at low doses and potentiated them at higher doses. Stress augmented the dopaminergic damage in the caudate, accumbens and amygdala. The interaction was strongest in the amygdala. Major findings are (1) stress has dose-dependent effects on the motor ability response to MPTP, (2) stress potentiates the neurochemical deficits produced by MPTP and (3) the amygdala is the most sensitive of the three areas to the stress-MPTP interaction. Supported by WSU Research Challenge Award.

J. B. Lucot[1], M. Dubovicky[1,2], J.R. Wells[1]. Effect of Pyridostigmine and Chronic Shaker Stress on Acoustic Startle Response, Pre-pulse Inhibition and Open Field Behavior of Mice

[1]Wright State University, School of Medicine, Dayton, OH; [2]Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia

Pyridostigmine bromide (PB) was used to protect soldiers from nerve agents. Chronic stress may alter the blood brain barrier (BBB) and allow toxins or drugs to enter the brain. We evaluated the effect of PB dosing during chronic intermittent shaker stress (CISS) on acoustic startle response (ASR), prepulse inhibition (PPI) and open field (OF) behavior of mice. Mice were treated with PB at the doses of 0 or10 mg/kg/day. One half of mice were exposed to shaker stress for 7 days (2 min shakings separated by an average of 30 min rest). The others were housed in standard conditions. Mice were tested in the startle monitor system for ASR and PPI. The system was set up for 6 types of stimulus trials: no stimulus (background, 60 dB), prepulse (70 dB), pulse (100 dB and 120 dB), prepulse plus pulse (70 dB+100dB and 70 dB+120 dB). The mice were tested during the CISS on days 2 and 7 (S2, S7) and 7, 14, 21 and 28 days after ending CISS. Another group was tested in the open field in 15 min sessions on day 1, 3 and 6 during CISS. The ASR for 120dB was significantly increased in the PB group on day S2 and S7 compared to SH controls. The ASR for 100dB was significantly higher in the PB group only when tested on day S2. Open field test revealed a significant decrease in locomotion in the PB group compared to SH controls on day 1 of treatment. There was no significant effect of coexposure of PB and chronic stress on the ASR and OF behavior.
Supported by DoD, contract No. 99214005.

James N. McDougal. Molecular Changes in Skin Following Acute Dermal Exposures to Irritating Chemicals. Pharmacology & Toxicology, Wright State University School of Medicine

Submitted to the Bioscience Meeting:

Many products and chemicals cause irritation when they contact the skin. Whole animal primary irritation testing (Draize test) has been around since 1944 with many modifications and variations. Because chemicals diffuse through the skin at different rates and have different irritant potencies, there is an exposure duration for most substances that would not cause irritation. Only minutes of contact could be safe for some substances but it might take hours or days for other substances to cause irritation. The general purpose of these studies is to characterize the biological cascade in the skin that results from acute chemical exposure. Ultimately our goal is to develop a biologically based model of irritation that can be used to predict safe exposure durations for a wide variety of compounds. We exposed rats, in vivo, to irritating chemicals for one hour and investigated the temporal changes in gene expression. Traditional histopathology and immunohistochemistry were also used to compare response of the skin to the fuel and solvents. We found that the parameters in the irritant cascade that we investigated responded differently depending on the degree of irritancy of each chemical. Measurements of protein levels will refine our preliminary understanding of this acute irritant cascade.

Ropp. S.[1], I. Bernatova[1,2], S.J.Paton[1], W.A. Price[1], M. Morris[1] and D.R. Cool[1]. **SELDI-TOF MASS SPECTROMETRY PROTEOMIC ANALYSIS OF PYRIDOSTIGMINE BROMIDE AND STRESS ON THE HP-AXIS.**

1.Department of Pharmacology & Toxicology, Wright State University, School of Medicine, Dayton, OH 45435

2. Cardiovascular Physiology, Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovakia.

Abstract submitted to the Society of Toxicology Meeting, 2002

Pyridostigmine bromide (PB) is an acetycholinesterase (AChE) inhibitor used as a preemptive treatment against possible exposure to organophosphate nerve agents and as a treatment for myasthenia gravis. In order to analyze the effects of PB and stress on the hypothalamic-pituitary axis (HP-axis), SELDI-TOF mass spectrometry proteomic analysis was conducted on the hypothalamus in parallel with AChE activity assays. Two models were used; PB-treatment in which doses of 0, 0.5, 1, 2 and 3 mg/kg/day were administered using Alzet minipumps implanted subcutaneously in the mice for 7 days or 7 day chronic shaker stress. The hypothalamic AChE activity in the 7-day chronic stress model was unchanged when compared to controls. PB (5 mM) was able to inhibit 98% of this activity. In contrast, a significant dose-dependent decrease in hypothalamic AChE activity (>40% at the highest dose of 3 mg/kg/day) was observed in the chronically-treated PB model. Proteomic analysis of the same stress and PB-treated hypothalamic homogenates by SELDI-TOF MS showed significant and similar changes in the hypothalamic peptide/protein profiles for both stressed and PB-treated mice. Ratio analysis of the protein profiles indicated a decrease in peak intensity for large (>4000 d) and small (<4000 d) peptides. However, the decrease in intensity was greater for the larger proteins (2.0-7.5 vs small, 1.3-2.0), suggesting a buildup of the smaller peptides within the cells. Analysis of the posterior pituitary also showed a similar decrease in arg-vasopressin peptide in both stress and PB-treated mice. These results suggest that stress and PB both affect the peptide protein profiles in the hypothalamus by changing either the protein expression or peptide processing and secretion patterns of the cells. Supported by DoD contract No.99214005.

Wells, J.R.[1], M. Dubovicky[1,2], J. B. Lucot[1]. Effects of shaker stress on the open field behavior of mice under diurnal and nocturnal conditions.
[1]Wright State University, School of Medicine, Dayton, OH; [2]Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia

Stress is considered to be an important risk factor in the development of physical and mental illnesses. In our study, we hypothesized that behavioral responses to stressful stimuli would be different when evaluated under different phases of circadian cycle. The goal of the study was to determine the effects of acute and chronic shaker stress on day/night patterns of behavioral responses of C57Bl/6J mice in the open field. Acutely stressed mice were shaken once for 15 minutes, while chronically stressed mice were shaken for 2 minutes with randomized rest intervals with a mean of 30 minutes for 7 days. The mice were tested in the open field in the light or dark phase of circadian cycle immediately after stress (day 1) and 7, 14 and 21 days after discontinuation of stress. Acute stress did not have any significant effect on the open field behavior of mice tested under either light conditions. Chronic stress resulted in a significant decrease of locomotor activity in the light phase that persisted up to 14 days after termination of stress. In the dark phase there was no significant effect of chronic shaker stress. In addition, mice tested in the dark phase exhibited less anxiety-like behavior as expressed by central zone activities and had higher emotionality expressed by defecation rate. Results of the present study indicate an influence of the day/night cycle on long-term behavioral responses of mice to chronic shaker stress in the open field.

Project 2 Abstracts:

Bicknell, I. R., N. V. Reo, L. Prochaska, M. Forquer, L. Shroyer, A. Neuforth, and T. Young. "ABR, NMR, and energy metabolism studies of the effects of long-term exposure to low doses of DEET in the rat". Annual meeting of the Association for Research in Otolarynglogy (ARO), St. Petersburg Beach, FL, February 2002.

Despite investigations of the chemicals to which military personnel may have been exposed during the Gulf War, their role in the symptoms associated with the Gulf War syndrome is not clear. Effects of exposure to the insect repellent N,N diethyl-m-toluamide (DEET), in the presence and absence of noise stress, were evaluated in the rats by three different means: auditory brainstem response (ABR), nuclear magnetic resonance (NMR) and *in vitro* biochemical assays. ABR thresholds were determined using frequency-specific tone bursts and 100 □s clicks. Brain energy metabolites were assayed by monitoring the ratio of phosphocreatine to adenosine triphosphate by ^{31}P NMR. Relative levels of brain choline (Cho), creatine (CR), and N-acetylaspartate (NAA) were determined from proton NMR spectra. *In vitro* energy coupling ratios were assessed using succinate, pyruvate and malate as substrates.

Male Sprague-Dawley rats were injected intraperitoneally once weekly for four consecutive weeks with either DEET (225 mg/kg in arachis oil) or vehicle (controls). Some animals were exposed immediately after each injection to 8 hr of white noise at 85 dBSPL. ABRs and NMRs were done on anesthetized rats 24 hrs before the initial injection and at post-initial-injection times of 24 hrs (ABR), week 1 (ABR), week 2 (ABR, NMR), week 3 (ABR), and week 4 (ABR, NMR). After the final ABR and NMR measurements, rats were euthanized, mitochondria were isolated from brainstems, and respiratory chain function was assessed.

No significant time-related changes in ABR thresholds were observed nor were major noise-related effects seen. A repeated measures ANOVA ($p<0.05$) indicated no statistically significant changes in *in vivo* NMR measures of brain energy metabolites. Cho:Cr and NAA:Cr ratios were not significantly different (DEET-treated vs. control). No differences in respiratory control ratios, cytochrome oxidase activity, or succinate dehydrogenase activities were observed. *A* 15% inhibition of NADH dehydrogenase activity (DEET vs. control) was observed in vitro.

Project 4 Abstracts:

Steven J. Berberich, Ph.D., Molly McGorry, John Paietta, Ph.D. and Madhavi Kadakia, Ph.D. Chemical Warfare Agent: Toxicogenomics Conference, US Army Medical Research and Material Command, Maryland, November 2001, "Expression Profiling of Pyridostigmine Bromide responsive genes in Cultured Human (HCN-2) Cortical Neurons."

Gene Expression Laboratory and Department of Biochemistry & Molecular Biology, Wright State University, Dayton, OH USA

With the ever expanding knowledge and sequencing of genomes, DNA microarrays have evolved as invaluable tools. Microarrays provide a quantitative, as well as qualitative, analysis of gene expression simultaneously across entire genomes. In this study, we have been examining changes of gene expression in cultured human cortical neurons (HCN-2) exposed to pyridostigmine bromide. Pyridostigmine is a reversible inhibitor of acetylcholinesterase administered to soldiers during the Persian Gulf War as a pretreatment against possible chemical warfare threat due of its protective effect against organophosphate poisoning. Increased frequency of central nervous system symptoms has been shown to be associated with PB possibly due to brain penetration as a result of stress. Phase I of this study has focused on demonstrating effective differentiation of the HCN-2 cortical cells into mature neurons based on monitoring gene expression in undifferentiated verses differentiated HCN-2 cells as well as morphological studies. Gene expression analyses demonstrated that a set of 111 and 85 genes were consistently up- or down-regulated, respectively following differentiation of HCN2 as determined by stringent data mining analyses (self organizing map, increased difference call and Mann U Whitney). Genes upregulated consisted of those involved in extracellular matrix and cytoskeleton formation, signal transduction and a variety of transcription factors. Of particular interests were genes involved in neuronal development and functions. The second phase of the study involved examining the gene expression profiles of differentiated HCN-2 cells following treatment with millimolar concentrations of pyridostigmine bromide for 24-72 hours. While no statistically significant changes in gene expression were observed following 24 hours of pyridostigmine bromide treatment, significant changes were observed following longer periods of treatment. Interestingly, a subset of genes induced following 48 hours of pyridostigmine bromide treatment were found repressed after 72 hours of pyridostigmine treatment. Overall, there was no single gene that showed a consistent pattern of gene alteration following pyridostigmine bromide. Future phases of this project will involve examining gene expression profiles of differentiated HCN-2 cells following sarin treatment or following combinations of pyridostigmine bromide and sarin.

Steven J. Berberich, Ph.D., Molly McGorry, Phylip Chen, John Paietta, Ph.D. and Madhavi. 2002 EPA/ARFL Toxicology Meeting, Cincinnati, OH March 2002, "GeneChip Expression Profiling of Cultured Human Neurons exposed to Pyridostigmine Bromide" Kadakia, Ph.D.

Gene Expression Laboratory and Department of Biochemistry & Molecular Biology, Wright State University, Dayton, OH USA

With the ever expanding knowledge and sequencing of genomes, DNA microarrays have evolved as invaluable tools. Microarrays provide a quantitative, as well as qualitative, analysis of gene expression simultaneously across entire genomes. In this study, we have been examining changes of gene expression in cultured human cortical neurons, HCN-2 and SHSY5Y exposed to pyridostigmine bromide. Pyridostigmine is a reversible inhibitor of acetylcholinesterase administered to soldiers during the Persian Gulf War as a pretreatment against possible chemical warfare threat due of its protective effect against organophosphate poisoning. Increased frequency of central nervous system symptoms has been shown to be associated with PB possibly due to brain penetration as a result of stress. Phase I of this study has focused on demonstrating effective differentiation of the HCN-2 cortical cells into mature neurons based on monitoring gene expression in undifferentiated verses differentiated HCN-2 cells as well as morphological studies. Gene expression analyses demonstrated that a set of 111 and 85 genes were consistently up- or down-regulated, respectively following differentiation of HCN2 as determined by stringent data mining analyses (self organizing map, increased difference call and Mann U Whitney). Genes upregulated consisted of those involved in extracellular matrix and cytoskeleton formation, signal transduction and a variety of transcription factors. Of particular interests were genes involved in neuronal development and functions. The second phase of the study involved examining the gene expression profiles of differentiated HCN-2 cells following treatment with millimolar concentrations of pyridostigmine bromide for 24-72 hours. Only a few statistically significant changes in gene expression have been observed following pyridostigmine bromide treatment. The current phase of the project has been to determine the gene expression profiles of SHSY5Y neurons following treatment with pyridostigmine bromide. Future phases of this project will involve examining gene expression profiles of human neuronal cells following treatment with the nerve agent sarin or following exposure to combinations of pyridostigmine bromide and sarin.